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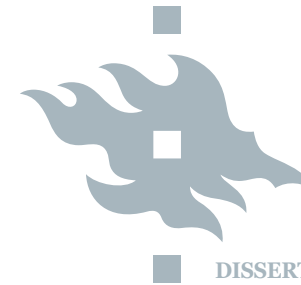
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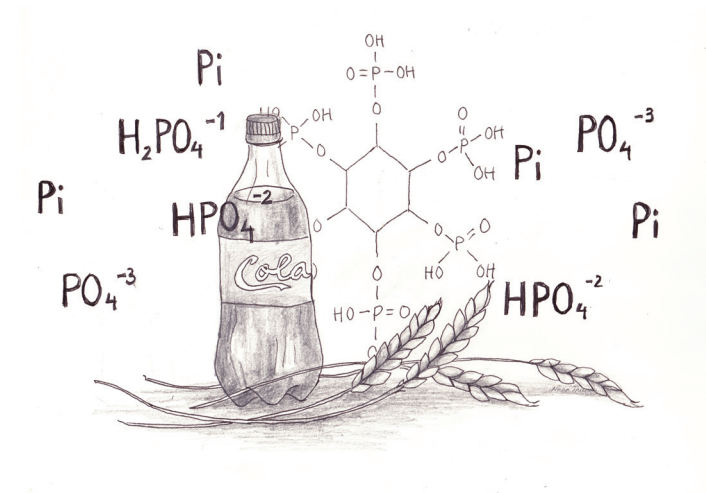
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Pain and Associated Procedural Anxiety in Adults Undergoing Bone Marrow Aspiration and Biopsy – Therapeutic Efficacy and Feasibility of Various Analgesics



SUVI ITKONEN

## Dietary Phosphorus – Bioavailability and Associations with Vascular Calcification in a Middle-Aged Finnish Population



DEPARTMENT OF FOOD AND ENVIRONMENTAL SCIENCES  
FACULTY OF AGRICULTURE AND FORESTRY  
DOCTORAL PROGRAMME IN POPULATION HEALTH  
UNIVERSITY OF HELSINKI

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Department of Food and Environmental Sciences  
University of Helsinki  
Helsinki

**Dietary phosphorus  
– Bioavailability and Associations with Vascular  
Calcification in a Middle-Aged Finnish Population**

**Suvi Itkonen**

**ACADEMIC DISSERTATION**

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*To those who did not survive*

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## Tiivistelmä, Finnish summary

Fosforin saanti on länsimaissa kaksin-kolminkertaista suhteessa ravitsemussuosituksiin. Fosforin kokonaissaantia on kasvattanut fosforilisäaineiden käyttö elintarviketeollisuudessa ja on arvioitu, että jopa 10-50 prosenttia fosforin saannista on peräisin lisäaineista. Elintarvikekoostumustietokannoissa reseptipohjaisten elintarvikkeiden fosforipitoisuudet on laskettu raaka-aineiden pitoisuuksien perusteella, jolloin ne eivät perustu kemiallisesti analysoituihin tietoihin. Tällöin lisäaineiden osuutta fosforipitoisuuksissa ei välttämättä ole otettu huomioon. Fosforin todellisen saannin arviointia vaikeuttaa myös fosforin hyväksikäytettävyyden vaihtelevuus eri fosforilähteiden välillä; lisäaineperäinen epäorgaaninen fosfori imeytyy elimistöön paremmin kuin elintarvikkeen luontainen, orgaaninen fosfori. Munuaistautipotilailla runsaan fosforin saannin haitallisuus on ollut tiedossa jo pitkään, ja fosforirajoitteista ruokavaliota käytetään osana heidän hoitoaan, jotta mm. verisuonten kalkkeutumista voitaisiin estää; kalkkeutumisessa kalsiumfosfaattia saostuu suonten seinämiin. Viime vuosina on saatu tutkimustuloksia seerumin kohonneen fosfaattipitoisuuden haittavaikutuksista sydän- ja verisuonitautien riskiin ja verisuonten kalkkeutumiseen myös terveellä väestöllä, mutta ravinnon fosforin osalta tutkimustietoa on niukasti. Tämän väitöskirjatyön tavoitteena on valottaa runsaan fosforin saannin mahdollisia terveysriskejä Suomessa tuottamalla tietoa elintarvikkeiden fosforin hyväksikäytettävyydestä ja runsaan fosforin saannin yhteyksistä sydän- ja verisuonitautien riskitekijöihin väestötasolla.

Väitöskirjan ensimmäisessä osatyössä kehitettiin *in vitro* -analyysimenetelmä liukoisen, ns. elimistöön imeytyvän ja hyväksikäytettävissä olevan, fosforin pitoisuuksien määrittämiseen elintarvikkeista sekä analysoitiin liukoisen ja kokonaisfosforin määriä eri tavoin käsitellyistä ruis-, vehnä- ja ohranäytteistä. Hypoteesina oli, että viljatuotteiden prosessointi lisää liukoisen fosforin määrää. Analyysimenetelmästä johtuva laskennallinen epävarmuus vaikutti liukoisen fosforin pitoisuuksiin näytteissä suhteellisen vähän. Hapatetuissa viljatuotteissa liukoisen fosforin määrä oli suurempi kuin hapattamattomissa, myös pitkä kypsennysaika lisäsi liukoisen fosforin pitoisuuksia. Tämä johtunee viljojen



sisältämän fosforipitoisen fytaatin pilkkoutumisesta käsittelyn aikana, jolloin siitä vapautuu liukoista, epäorgaanista fosforia.

Toisessa osatyössä hyödynnettiin ensimmäisessä osatyössä kehitettyä menetelmää liukoisen fosforin pitoisuuksien määrittämiseen valikoiduista kasvipohjaisista elintarvikkeista ja juomista. Myös kokonaisfosforipitoisuudet määritettiin ja liukoisen fosforin pitoisuuksia verrattiin niihin. Hypoteesina oli, että lisäainefosforia sisältävissä tuotteissa liukoisen fosforin osuudet ovat suhteellisesti korkeampia kuin tavanomaisissa tuotteissa. Tutkimuksessa havaittiin, että analysoidut kasvipohjaiset elintarvikkeet sisälsivät vaihtelevia määriä fosforia, mutta suurin osa siitä ei ollut liukoista lukuun ottamatta lisäainefosforia sisältäviä tuotteita. Lisäainefosforia sisältävissä tuotteissa, kuten kolajuomissa ja leivinjauheella kohotetuissa muffinseissa, oli suurempi liukoisen fosforin osuus verrattuna tavanomaisiin tuotteisiin.

Kolmannessa osatyössä tutkittiin poikkileikkausasetelmalla keski-ikäisillä miehillä ja vaihdevuosi-ikää edeltävillä naisilla fosforin kokonaissaannin, ravinnon fosforitiheyden (fosforin saanti suhteutettuna energian saantiin) sekä lisäainefosforin saannin yhteyksiä sydän- ja verisuonitautien riskitekijään, kaulavaltimon seinämän paksuuteen (n=546). Lisäainefosforin saantia tutkittiin ruoankäytön frekvenssikyselylomakkeella, muut ruoankäyttötiedot kerättiin kolmen vuorokauden ruokapäiväkirjanpidolla. Hypoteesina oli, että runsas fosforin saanti, etenkin lisäainefosforin muodossa, on yhteydessä verisuonten kalkkeutumista kuvaavaan paksumpaan kaulavaltimon seinämään ja täten suurentuneeseen sydän- ja verisuonitautien riskiin. Tutkimuksessa havaittiin lineaarinen yhteys ravinnon fosforitiheyden ja lisäainefosforin saannin sekä kaulavaltimon seinämän paksuuden välillä koko aineistossa, ja naisilla lisäainefosforin saannin ja kaulavaltimon seinämän paksuuden välillä. Miehillä tilastollisesti merkitseviä tuloksia ei ollut havaittavissa. Tulosten perusteella runsas fosforin saanti, etenkin lisäainefosforin muodossa, saattaisi olla yksi sydän- ja verisuonitautien riskitekijöistä myös väestötasolla, ei ainoastaan munuaistautipotilailla.

Väitöskirjan tulokset tukevat aiempaa käsitystä siitä, että fosforilähteellä on merkitystä arvioitaessa fosforin saantia väestötasolla. Kasvikunnan tuotteet

yleisesti sisältävät vähäisiä määriä liukoista fosforia mutta lisäänefosforipitoiset kasvipohjaiset elintarvikkeet sisältävät suhteellisesti suurempia liukoisen fosforin pitoisuuksia verrattuna tavanomaisiin tuotteisiin. Tulosten perusteella liukoisen fosforin määrittäminen elintarvikkeista on luotettava mutta tarvitsee vertailuarviointia *in vivo* -analyysimenetelmän kanssa ennen kuin tuloksia voidaan yleistää. Vaikka runsaan fosforin saannin (ravinnon fosforitiheys ja lisäänefosforin saanti) havaittiin olevan yhteydessä yhteen sydän- ja verisuonitautien riskitekijään, kaulavaltimon seinämän paksuuteen, poikkileikkaustutkimusasetelman perusteella syy-seuraussuhteita ei kuitenkaan voida päätellä. Tämän vuoksi etenkin hyvin imeytyvän lisäänefosforin mahdollisia haittavaikutuksia sydän- ja verisuoniterveyteen pitäisi tutkia tarkemmin sekä interventio- että seurantatutkimuksilla ennen kuin runsaan fosforin saannin mahdollisesta haitallisuudesta väestötasolla voidaan tehdä lopullisia johtopäätöksiä. Tämän selvittämiseksi tarvitaan lisää tietoa myös fosforilisäaineiden käytöstä elintarviketeollisuudessa sekä päivitettyjä analyysitietoja elintarvikkeiden fosforipitoisuuksista.

AVAINSANAT: elintarvikelisiäaineet, elintarvikkeet, fosfaatti, fosfori, hyväksikäytettävyys, kasvipohjaiset elintarvikkeet, kaulavaltimon seinämän paksuus, liukoinen fosfori, sydän- ja verisuonitaudit, verisuonten kalkkeutuminen, viljatuotteet

## Abstract

Phosphorus (P) intake in Western countries exceeds the nutritional recommendations 2- to 3-fold, and the increased use of food additive phosphates (FAPs) in the food industry has augmented total P (TP) intake; an estimated 10-50% of TP intake comes from additives. Difficulties in measuring true P intake occur because bioavailability of P differs between foodstuffs; inorganic P from additives is absorbed better in the intestine than natural, organic P. Moreover, in food composition databases, P contents of the recipe-based foodstuffs are calculated based on nutrient contents of raw materials, and not on chemically analyzed values. Thus, the amounts of FAPs may have not been taken into account. It has been stated that all P sources cannot be defined similarly as detrimental to health due to differing absorbability and bioavailability. The harmfulness of high P intake to kidney patients has been known for years, and dietary P restriction is used in the treatment of the disease, to avoid vascular calcification, which occurs due to calcium-phosphate deposition in the vascular vessel wall. However, recently, concerns have been raised about the role of elevated serum phosphate concentrations in cardiovascular health in the general population, but data on dietary P is scarce. This thesis aims to offer new insights into the potential health risks related to high dietary P intake in Finland by providing knowledge on the bioavailability of P in foodstuffs, and on the association of high dietary P intake with cardiovascular disease risk factors in the general population.

In Study I, a new method was developed for analysis of *in vitro* digestible P (DP), indicating bioavailable and absorbable P, and *in vitro* DP and TP contents of certain differently processed rye, wheat and barley samples were determined. The hypothesis was that processing of cereals increases the content of DP. The calculated uncertainty of the analysis method had little effect on the DP contents in the samples. Soured cereals contained more DP than unsoured cereals, and the long processing time increased the amounts of DP. This probably is due to the degradation of P-containing phytate, which releases digestible, inorganic P.

In Study II, the DP contents of selected plant-based foodstuffs and beverages were determined by the analysis method developed in Study I. Also TP contents were analyzed, and DP contents in the same foods were compared to them. The hypothesis was that the proportion of DP is lower in plant-based FAP-free products than in products containing FAPs. The analyzed plant-based products contained varying amounts of P, but most P was not absorbable, except in FAP-containing foodstuffs. The FAP-containing products, especially cola drinks and baking powder-leavened muffins, had higher amounts of DP relative to FAP-free products.

In Study III, a cross-sectional design was used to investigate the associations between TP intake, P density of the diet (TP intake related to energy intake), FAP intake, and a risk factor of cardiovascular disease, carotid intima-media thickness in middle-aged males and premenopausal females (n=546). Data on FAP intake were collected by food frequency questionnaires, and nutrients other than FAP were assessed by 3-day food records. Hypothesis was that high dietary P intake, especially in the form of FAP, is associated with vascular calcification in terms of high carotid intima-media thickness and thus, with increased risk of cardiovascular diseases. Here, linear associations among P density of the diet, FAP intake, and carotid intima-media thickness were found when all subjects were included in the analysis, and between FAP intake and carotid intima-media thickness in females. However, among males no significant associations were found. These findings suggest that high dietary P intake, especially in the form of FAP, could be a cardiovascular risk factor not only in renal patients but also in the general population.

In conclusion, results of this thesis reinforce the previous understanding about the importance of the source of P when assessing P intake in a population. The plant-based foodstuffs generally contain low amounts of absorbable P, but the FAP-containing plant-based products have relatively higher absorbable P contents than FAP-free products. The method developed for the analysis of DP is reliable but requires validation against an *in vivo* method before these results can be generalized. Albeit high dietary P intake (P density of the diet and FAP intake) was associated with a cardiovascular disease risk factor, carotid intima-media thickness, due to the cross-sectional design of the study, no causal

relationships can be inferred. Thus, the potential adverse effects of highly absorbable FAPs should be further investigated both in intervention and in follow-up studies before final conclusions about the harmfulness of high P intake for the general population can be drawn. To find this out, data on use of FAPs in the food industry and updated information on P contents in foodstuffs are also needed.

**KEY WORDS:** bioavailability, cardiovascular diseases, carotid intima-media thickness, cereals, digestible phosphorus, food additives, foodstuffs, phosphate, phosphorus, plant-based foodstuffs, vascular calcification

## List of original publications

This thesis is based on the following publications, referred to in the text by their Roman numerals (I-III):

I: **Itkonen ST**, Ekholm PJ, Kemi VE, Lamberg-Allardt CJE. Analysis of in vitro digestible phosphorus content in selected processed rye, wheat and barley products. *J Food Comp Anal* 2012;25:185-189.

II: Karp H, Ekholm P, Kemi V, **Itkonen S**, Hirvonen T, Närkki S, Lamberg-Allardt C. Differences among total and in vitro digestible phosphorus content of plant foods and beverages. *J Ren Nutr* 2012;22:416-422.

III: **Itkonen ST**, Karp HJ, Kemi VE, Kokkonen EM, Saarnio EM, Pekkinen MH, Kärkkäinen MUM, Laitinen EKA, Turanlahti MI, Lamberg-Allardt CJE. Associations among total and food additive phosphorus intake and carotid intima-media thickness – A cross-sectional study in a middle-aged population in Southern Finland. *Nutr J* 2013;12:94.

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### Contribution of the authors to Studies I-III

I: PJE designed the analyses and STI performed the sampling. STI conducted the P analyses. STI analyzed and interpreted the data. STI drafted the manuscript with the help of PJE. All authors read, reviewed and approved the final manuscript.

II: HK, PE, VK, TH and CLA planned the study. SN carried out the laboratory analysis. HK, PE, and SI analyzed the data. HK wrote the manuscript with PE and TH, and SI and the other co-authors critically reviewed the paper.

III: STI, HJK, VEK, and CJELA designed the study. MIT designed the IMT measurement. STI, HJK, VEK, EMS, MHP, and MUMK collected the data. STI and EMK analyzed the data. STI, CJELA, and EMK interpreted the data. STI drafted the manuscript with the help of CJELA. STI, EMK, and CJELA are responsible for the integrity of data analysis. All authors read, reviewed and approved the final manuscript.

## Abbreviations

1,25(OH)<sub>2</sub>D 1,25-hydroxy vitamin D, calcitriol  
25(OH)D 25-hydroxy vitamin D, calcidiol  
ANCOVA analysis of covariance  
ANOVA analysis of variance  
BMI body mass index  
Ca calcium  
CaPi calcium-phosphate  
cIMT carotid intima-media thickness  
CKD chronic kidney disease  
CRP C-reactive protein  
C-terminal FGF23 carboxy-terminal fibroblast growth factor 23  
CV% coefficient of variation percentage  
CVD cardiovascular disease  
CYP2R1 cytochrome P 450 2R1  
CYP24A1 cytochrome P 450 24A1  
CYP27B1 cytochrome P 450 27B1  
DP digestible phosphorus  
EFSA European Food Safety Authority  
eGFR estimated glomerulus filtration rate  
FAP food additive phosphate  
FFQ food frequency questionnaire  
FGF23 fibroblast growth factor 23  
GFR glomerulus filtration rate  
HDL high-density lipoprotein  
HDL-C high-density lipoprotein cholesterol  
HNO<sub>3</sub> nitric acid  
hs-CRP high-sensitivity C-reactive protein  
iCa ionized calcium  
iPTH intact parathyroid hormone  
ICP-AES inductively coupled plasma -atomic emission spectrometry  
ICP-MS inductively coupled plasma -mass spectrometer  
ICP-OES inductively coupled plasma -optical emission spectrometer  
IMT intima-media thickness  
LDL low-density lipoprotein  
LDL-C low-density lipoprotein cholesterol  
Mg magnesium  
Na sodium  
NaPi 2a sodium-dependent phosphate cotransporter 2a  
NaPi 2b sodium-dependent phosphate cotransporter 2b  
NaPi 2c sodium-dependent phosphate cotransporter 2c  
P phosphorus  
Pi phosphate  
PTH parathyroid hormone  
P-Pi plasma phosphate  
S-Pi serum phosphate  
TP total phosphorus

# 1. Introduction

The Western population is aging and the incidence of many diseases is on the rise. Cardiovascular diseases (CVD) are leading causes of mortality and morbidity worldwide (O’Leary and Bots 2010), and the incidence of renal disease is also increasing as an adjuvant disease of type 2 diabetes (Niemi and Winell 2005). Traditional risk factors for CVD are well known, but concerns have recently been raised on the role of high dietary phosphorus (P) intake in CVD. Especially in patients with chronic kidney disease (CKD), high dietary P intake has been shown to be deleterious, and high serum phosphate (S-Pi) concentrations in this patient group are associated with a higher risk of mortality (Qunibi et al. 2002). CKD patients are unable to excrete P properly, thus, they are treated with dietary P restriction (Qunibi et al. 2002). Subsequent research data have revealed associations between high S-Pi concentrations and higher risk of cardiovascular diseases and mortality also in the general population (Tonelli et al. 2005, Foley et al. 2009, Ruan et al. 2010).

P intake in Western countries exceeds 2- to 3-fold (Calvo 1993, Welch et al. 2009, Helldán et al. 2013a, Massé et al. 2014) the nutritional recommendations (600-700 mg/d) (Food and Nutrition Board 1997, Nordic Council of Ministers 2013). Results on the detrimental effects of high P intake on bone metabolism have been reported (Kärkkäinen and Lamberg-Allardt 1996, Kemi et al. 2006, 2008, 2009, 2010), but information on high P intake and CVD in the general population is scarce. Experimental work has shown that high dietary P intake induces vascular calcification; deposition of calcium-phosphate (CaPi) complexes in the vascular vessel wall (Uribarri and Calvo 2013). High P intake has also caused the left ventricular hypertrophy of the heart (Yamamoto et al. 2013) and has acutely impaired the endothelial function of the vascular system (Shuto et al. 2009). A recent study of the NHANES III population showed that high P intake was associated with increased all-cause mortality (Chang et al. 2014a).

Dietary P is an important element in energy metabolism, in phosphorylation of proteins, in maintaining the acid-base equilibrium, and in bone as a component of hydroxyapatite. Almost all foodstuffs contain P, but the intake has mainly



increased as a consequence of the expanding use of food additive phosphates (FAP) in the food industry (Suurseppä et al. 2001, Winger et al. 2012). Carrigan et al. (2014) estimated that 10-50 % of total P (TP) intake in Western countries comes from FAPs, and intake values of up to 1000 mg/d have been presented (Winger et al. 2012). Bioavailability of P differs between foodstuffs; in cereals and legumes P is in the form of phytic acid and thus, not soluble until degraded in food processing (Sandberg 2002). However, more than 90% of P from FAPs is absorbed by the human body (Sullivan et al. 2007). When assessing P intake, some difficulties exist: natural P and FAP cannot be distinguished analytically (Suurseppä et al. 2001); thus, the P contents of foodstuffs in food composition databases are reported as TP. Moreover, in food composition databases, P contents of the recipe-based foodstuffs are calculated based on nutrient contents of raw materials, and not on chemically analyzed values. Thus, the amounts of FAPs may have not been taken into account (Oenning et al. 1988, Sullivan et al. 2007).

Thus, a new method is needed to assess *in vitro* the digestible P (DP) (corresponding to the bioavailable P) in foodstuffs. Because of raised concerns on potential adverse health effects of high P intake and elevated S-Pi concentrations (Tonelli et al. 2005, Kemi et al. 2006, Huttunen et al. 2006, 2007, Foley et al. 2009, Shuto et al. 2009, Ruan et al. 2010), the scope of this study was widened to include the normal population and habitual dietary P intake as TP and FAP. A new analytical method is developed for measurement of the *in vitro* DP contents in foodstuffs, and analysis of DP in differently processed cereals is carried out (Study I), as well as the differences between TP and *in vitro* DP content in selected popular P-containing plant-based foodstuffs (Study II) are examined. Moreover, associations between dietary P and cardiovascular health in terms of carotid intima-media thickness (IMT) are discussed (Study III).

## 2. Review of the literature

### 2.1. Phosphorus in human nutrition

#### 2.1.1. Dietary intake and recommendations

The National Nutrition Council recommends a P intake of 600 mg/d for adults of both sexes (National Nutrition Council 2014), based on Nordic Nutrition Recommendations (Nordic Council of Ministers 2013) (Table 1). The upper limit of daily P intake is 5000 mg/d (National Nutrition Council 2014). In the United States, the estimated average requirement (except for adolescents) is 400 mg/d, and the recommended daily intake is 700 mg/d (Food and Nutrition Board 1997). The European Food Safety Authority produced a report in 2005 concerning the potential adverse effects of high P intake, but made no recommendations based on these effects (EFSA 2005).

**Table 1.** *Recommended phosphorus (P) intakes in different age groups*<sup>1</sup>.

Age	Recommendation (P mg/d)
<6 months	-
6-11 months	420
12-23 months	470
2-5 years	470
6-9 years	540
10-13 years	700
14-17 years	700
18-20 years	700
Adults >20 years	600
Pregnant women	700
Breastfeeding women	900

<sup>1</sup>National Nutrition Council 2014

Dietary P intake in Western countries exceeds 2- to 3-fold (Calvo 1993, Welch et al. 2009, Helldán 2013a) the nutritional recommendations (600-700 mg/d) (Food and Nutrition Board 1997, Nordic Council of Ministers 2013). Mean intake of P in the National FINDIET 2012 Survey was among females 1369 mg/d and among males 1694 mg/d, being lower in the older age groups (Helldán et al. 2013a). In Finland, the most important sources of P are dairy products (males 36%, females 35% of P intake), cereal products (males 20%, females 24%) and meat products (males 17%, females 14%) (Helldán et al. 2013a). Table 2 shows the approximate mean P intake ranges in the European countries that participated in the EPIC study (Welch et al. 2009). When comparing mean dietary P intakes in Finland from 2002 to 2012, the mean intakes have decreased (males: from 1928 to 1694 mg/d; females from 1465 to 1369 mg/d), however, the different assessment methods (3-day food records vs. 48-h food recalls) may impair the comparability between the results (Kleemola et al. 1994, Helldán et al. 2013a).

**Table 2.** *Approximate ranges of phosphorus (P) intakes stratified by gender in the EPIC study in European countries (based on Welch et al. 2009).*

<i>Country</i>	<b>Approximate range of P intake (mg/d)</b>	
	<i>Females</i>	<i>Males</i>
Greece	1500	2100
Spain	1150-1400	1550-1900
Italy	1100-1200	1500-1700
France	1300	-
Germany	1100-1200	1500
The Netherlands	1350-1500	1800
United Kingdom	1200-1300	1400-1600
Denmark	1400-1500	1800
Sweden	1200-1300	1500-1650
Norway	1350	2100

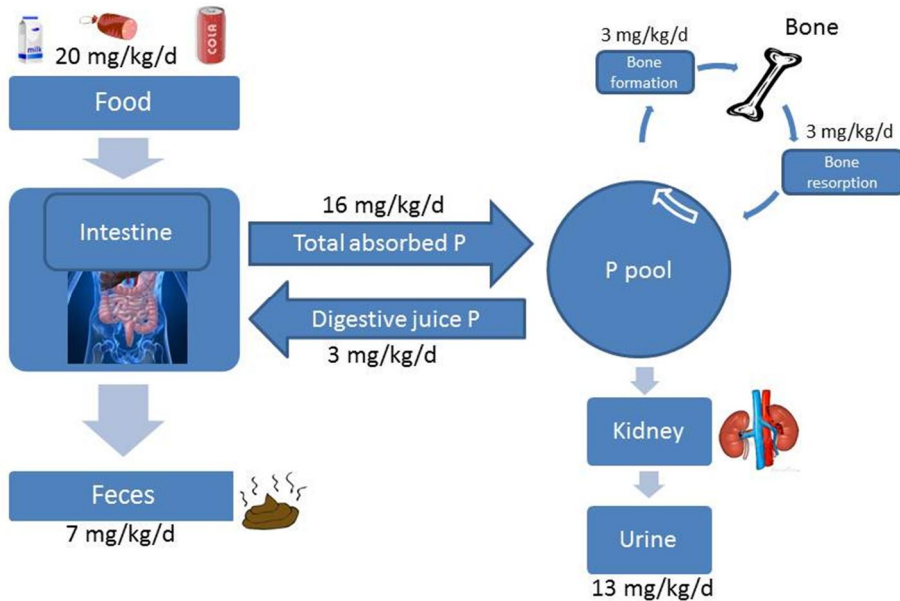
### **2.1.2. Functions of phosphate in the body**

P, in the form of phosphate (Pi), is required in several biological processes in the human body. Pi participates in the energy metabolism as a component of

adenosine triphosphate, creatinine Pi and other phosphorylated compounds such as nucleic acids (Knochel 2006). For instance, some hormones need Pi for the phosphorylation process. Pi exists also as a part of phospholipids in membranes, it acts as a buffer in acid-base homeostasis on the bone surface and regulates proton balance in the kidneys (Knochel 2006). An adult human body contains approximately 700 g P (Gaasbeek and Meinders 2005). Most of this, i.e. 60%, occurs as hydroxyapatite in the bone. A further 9% is in skeletal muscles, 10.9% in internal organs, and 0.1% in the extracellular matrix. In blood, Pi occurs in both organic and inorganic forms. The organic form comprises carbohydrates, lipids, and proteins. Of inorganic Pi, 85% occurs as free Pi ions ( $\text{HPO}_4^{-2}$ ,  $\text{H}_2\text{PO}_4^{-1}$  ja  $\text{PO}_4^{-3}$ ), 10% is bound to proteins, and 5% exists as a component of calcium- (Ca), magnesium- (Mg) and sodium- (Na) bound compounds (Gaasbeek and Meinders 2005).

### **2.1.3. Phosphorus balance**

An adult weighing 70 kg gets about 1600 mg P daily from the diet, and of this, about 40-80 % is absorbed in the intestinal tract, and about 1 g is excreted in the urine. The kidneys are the main regulator of P balance in the body (Heaney 2004, Bergwitz and Jüppner 2010). Absorption is regulated by parathyroid hormone (PTH), calcitriol (1,25-hydroxy vitamin D, i.e.  $1,25(\text{OH})_2\text{D}$ ), and fibroblast growth factor 23 (FGF23), the latter needing Klotho as its co-receptor. About 30% of P absorption is regulated by  $1,25(\text{OH})_2\text{D}$  (Bergwitz and Jüppner 2010) and the rest is absorbed in a paracellular fashion. Most of P is absorbed in the jejunum, where sodium-dependent phosphate cotransporter 2b (NaPi2b) mediates active intestinal P absorption, and is regulated by  $1,25(\text{OH})_2\text{D}$ . Absorbed P is stored in the skeleton (Bergwitz and Jüppner 2010). Figure 1 shows the balance between absorption and excretion of P.



**Figure 1.** *Phosphorus balance in humans (adapted from Berndt and Kumar 2007).*

P homeostasis interfaces with Ca homeostasis in the kidney (Peacock 2010). P reabsorption from urine in renal proximal tubules by type 2 and 3 sodium-dependent phosphate cotransporters (NaPi2 and NaPi3) is the main factor maintaining blood Pi homeostasis, and this is regulated by PTH and FGF23 (Bergwitz and Jüppner 2010). Pi is taken up into the cells from the circulation via these transporters. In addition, some other hormones, such as insulin, hormones on the somatotrophic pituitary axis, and possibly also fibroblast growth factor 7, matrix extracellular glycoprotein and secreted frizzled-related protein-4, seem to participate in P homeostasis, but their exact functions have yet to be elucidated (Bergwith and Jüppner 2010). When dietary P intake is high, FGF23 is secreted from bone to bloodstream. This suppresses secretion and synthesis of PTH, Pi reabsorption in the kidney, and production of 1,25(OH)<sub>2</sub>D (Kuro-o 2013). FGF23 works synergistically with PTH to increase renal Pi excretion by reducing expression of sodium-dependent phosphate

cotransporters 2a and 2c (NaPi2a and NaPi2c) in the proximal tubules (Bergwith and Jüppner 2010). PTH, which has receptors in the kidney, increases renal phosphate clearance and stimulates synthesis of 1,25(OH)<sub>2</sub>D (Bergwith and Jüppner 2010). However, the receptor that senses the S-Pi concentration has not yet been identified (Peacock 2010). Furthermore, dose response between S-Pi and FGF23 is much less rapid than, for instance, between Ca and its regulating hormones (Peacock 2010).

Pi is analyzed from serum or plasma as inorganic phosphate. The normal ranges for serum phosphate (S-Pi) concentrations are for females 0.85-1.50 mmol/l (corresponding to 2.63-4.64 mg/dl), for males ≤50 years of age 0.75-1.65 mmol/l (corresponding to 2.32-5.11 mg/dl), and for males ≥50 years 0.75-1.35 mmol/l (corresponding to 2.32-4.18 mg/dl) (Yhtyneet Medix Laboratoriot 2014). Normal Pi concentrations in children are higher than in adults, gradually lowering with age.

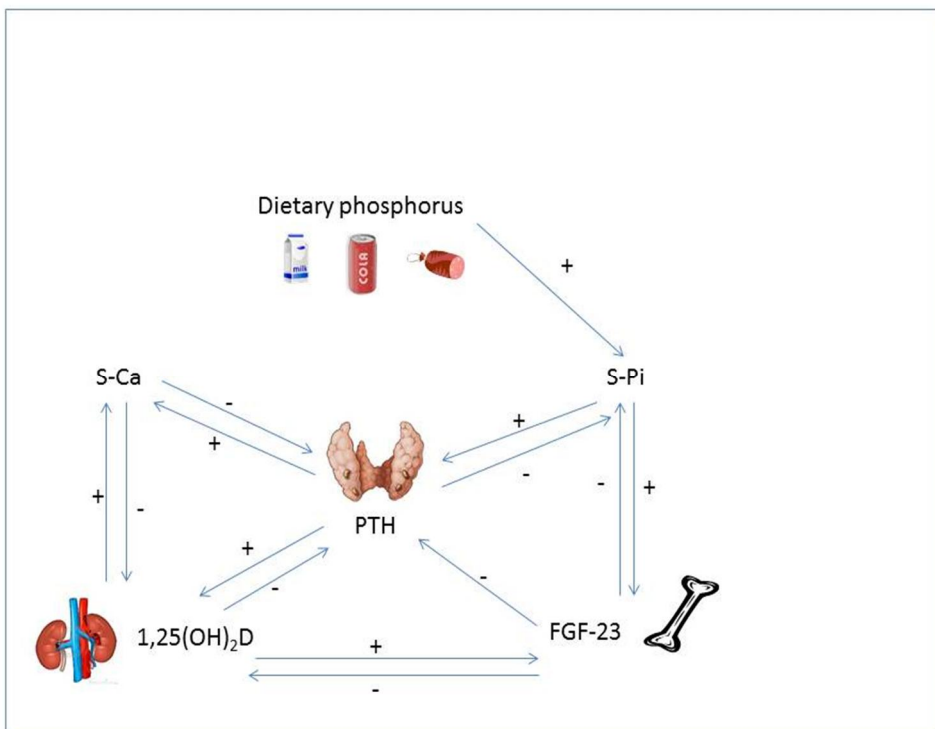
#### **2.1.4. Regulators of phosphate metabolism**

Many regulators participate in the Pi metabolism: PTH, FGF23 and its co-receptor Klotho, 1,25(OH)<sub>2</sub>D, Ca, secreted frizzled-related protein-4 and matrix extracellular glycoprotein. It is noteworthy that FGF23 and its contribution to Pi metabolism were unknown when many of the earlier studies on high P intake were done (Calvo and Tucker 2013); thus, newer studies have uncovered novel information on this topic. Figure 2 presents the regulators of P metabolism and their interactions.

#### ***Serum phosphate and serum calcium***

One percent of Pi in the human body is in serum, which is controlled to some degree by dietary P. Dietary P is known to have an impact on the biphasic circadian rhythm of S-Pi (Portale et al. 1987, Kärkkäinen and Lamberg-Allardt 1996). Changes in dietary P intake have an effect on the first phase of the rhythm pattern but independent of diet, the peak in S-Pi concentrations occurs

in the early morning (Portale et al. 1987). However, dietary P has only a small impact on S-Pi when measured after fasting or randomly, but increasing P intake acutely increases S-Pi (Portale et al. 1987, Kemi et al. 2006). Mean 24-h S-Pi concentrations and peak S-Pi after P load are proposed to be more useful than fasting S-Pi concentrations when assessing the effects of dietary P intake (Uribarri 2013). It is notable that in healthy adults and animals a high P diet for weeks can result in increased FGF23 or PTH without any perceivable changes in S-Pi (Uribarri and Calvo 2013). Increased Ca intake increases serum ionized Ca (iCa), which decreases S-PTH, leading to a lower excretion of P in urine (Mortensen and Charles 1996). Calcium sensing receptor reacts to elevated concentrations of S-Ca, inducing lowering of S-PTH concentrations. High S-Ca concentration stimulates FGF23 excretion and suppresses PTH secretion (Silver and Naveh-Many 2009).



**Figure 2.** Relationships between dietary phosphorus and regulator of metabolism. S-Pi = serum phosphate, S-Ca = serum calcium,  $1,25(\text{OH})_2\text{D}$  =

*calcitriol, PTH = parathyroid hormone, FGF-23 = fibroblast growth factor 23 (adapted from Silver and Naveh-Manly 2009).*

### ***Parathyroid hormone***

PTH is secreted from parathyroid glands (Bergwitz and Jüppner 2010). It regulates S-Pi, and elevated S-Pi stimulates PTH, probably by lowering extracellular Ca, and increasing stability of PTH messenger RNA. Continuous exposure to PTH induces bone resorption by activating osteoclasts but intermittent PTH increases bone formation by activating osteoblasts (Bergwitz and Jüppner 2010). PTH secretion is regulated by S-Pi and FGF23 and Klotho in the parathyroid gland. FGF23 is able to inhibit the synthesis of 1,25(OH)<sub>2</sub>D, which may negatively affect the secretion of PTH (Bergwitz and Jüppner 2010). However, PTH concentrations have been perceived to increase in response to increased S-Pi independently of S-Ca (Kärkkäinen and Lamberg-Allardt 1996). PTH is downregulated by increased S-Ca and 1,25(OH)<sub>2</sub>D and possibly by increased FGF23. PTH acts through G protein-coupled receptor PTHR1 to increase osteoblast activity (and thus, indirectly osteoclast activity) to inhibit renal Pi absorption, and to stimulate 1,25(OH)<sub>2</sub>D synthesis, which causes an increase in S-Ca and a decrease in S-Pi (Bergwitz & Jüppner 2010). PTH and FGF23 work synergistically to increase renal Pi excretion by reducing expression of NaPi2a and NaPi2c (Bergwitz and Jüppner 2010).

There is circadian variation in S-PTH concentrations, being lowest in the morning. Sex differences also appear to exist in the rhythm (Calvo et al. 1991). The rhythm is biphasic, as in S-Pi. PTH responds acutely to high dietary P load (Block et al. 2013), thus increasing urinary Pi excretion.

### ***Calcitriol, i.e. 1,25-dihydroxy vitamin D***

Vitamin D exists as cholecalciferol (vitamin D<sub>3</sub>) and ergocalciferol (vitamin D<sub>2</sub>). The former is produced in the skin after exposure to ultra-violet irradiation, normally sunlight, or received from the diet, the latter originates only from



plant-derived foods (Haussler et al. 2012). In the body vitamin D goes through 25-hydroxylation in the liver and is converted in the kidney to 1,25-dihydroxy vitamin D ( $1,25(\text{OH})_2\text{D}$ ), i.e. calcitriol, which is the active form of vitamin D (Bergwitz and Jüppner 2010). Circulating 25-hydroxy vitamin D ( $25(\text{OH})\text{D}$ ) describes the vitamin D status in the body. Cytochrome P 2R1 (CYP2R1) is responsible for 25-hydroxylation. Cytochrome P 27B1 (CYP27B1), the enzyme responsible for  $1-\alpha$ -hydroxylation in the kidney, is induced by calcitonin, PTH, hypocalcemia and hypophosphatemia. FGF23, hypercalcemia and hyperphosphatemia repress CYP27B1 expression (Haussler et al. 2012), and fibroblast growth factor 7 and secreted frizzled-related protein-4 seem to downregulate this synthesis. FGF23 and  $1,25(\text{OH})_2\text{D}$  also induce activity of renal cytochrome P 24A1 (CYP24A1) expression which converts  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$  into inactive metabolites (Haussler et al. 2012).

### ***Fibroblast growth factor 23 and Klotho***

FGF23 is the major regulator of renal Pi handling (Ketteler et al. 2013). FGF23, which needs Klotho as its co-receptor, is produced in osteocytes and osteoblasts (Ketteler et al. 2013) and secreted from bone by an unknown mechanism (Kuro-o 2013). Vitamin D receptor and  $1,25(\text{OH})_2\text{D}$  induce expression of FGF23 in bone, and increased S-Pi also accelerates the expression of FGF23. Dentin matrix protein 1 and phosphate-regulating gene with homologies to endopeptidases on the X chromosome suppress FGF23 production in bone, probably by indirect mechanisms (Bergwitz and Jüppner 2010). FGF23 is an attenuator of  $1,25(\text{OH})_2\text{D}$  action (Haussler et al. 2012).  $1,25(\text{OH})_2\text{D}$  and dietary P induce synthesis of FGF23 (Bergwitz and Jüppner 2010). FGF23 facilitates kidney Pi handling by decreasing expression of NaPi2a, NaPi2c and CYP27B1 in proximal tubules, thus, increasing urinary Pi excretion. Many organs have FGF receptors, but Klotho is expressed only in the distal tubulus, parathyroid gland chief cells and the choroid plexus of the brain. Klotho is needed to improve the affinity of FGF receptors to FGF23.

FGF23 seems to follow an inherent circadian rhythm, and both intact FGF23 and carboxy-terminal FGF23 (C-terminal FGF23) concentrations have been

shown to increase in humans after feeding a high P diet (Vervloet et al. 2011). Intact FGF23 is known to response more sensitively to dietary P load than C-terminal FGF23 (Burnett et al. 2006). It is also claimed that due to high dietary P load, FGF23 increases only when high P intake is continuous (Block et al. 2013, Uribarri and Calvo 2013).

All of these P metabolism-regulating hormones (parathyroid hormone, FGF-23, and 1,25(OH)<sub>2</sub>D) have been independently associated with adverse bone and CVD health aspects even when S-Pi concentrations have been in the normal range (Uribarri and Calvo 2013).

## **2.2. Sources and bioavailability of dietary phosphorus**

P occurs widely in foodstuffs, but the highest contents are in protein-rich foods, including meat, fish, eggs, dairy, legumes, whole-grain cereals, nuts and seeds (Kalantar-Zadeh et al. 2010). Inorganic Pi's and organic starch-based Pi's are used as food additives in various foodstuffs (Kalantar-Zadeh et al. 2010). Also some dietary supplements and some drugs contain P (Uribarri 2013). Tap water also contains P, albeit in very small amounts (EFSA 2005). P content of drinking water is legally restricted to 2.2 mg/l (European Union 1980). In foodstuffs P occurs in the form of Pi but nutritional recommendations and nutrient contents of foodstuffs are reported as P. Table 3 displays P contents of selected foodstuffs.

**Table 3.** *Phosphorus (P) contents of selected foodstuffs (Institution for Health and Welfare 2011).*

<b>Foodstuff</b>	<b>P (mg/100g)</b>	<b>Foodstuff</b>	<b>P (mg/100g)</b>
Milk, 1.5% fat	90	Beef, raw	172
Edam cheese	510	Pork, raw	158
Processed cheese, fat 27-35%	360	Smoked ham	200
Processed cheese, fat 5%	1570	Turkey cold cut	260
Oat flakes	430	Pizza minced meat, pork-beef	149.7
Oat porridge	54.3	Tomato	30
Rye bread	229.1	Orange, peeled	23
(rye flour, wheat flour)		Milk chocolate	300
Wheat flour	95	Cola drink	15
Eggwhite	11	Soft drink	2
Egg yellow	540	Apple juice	6

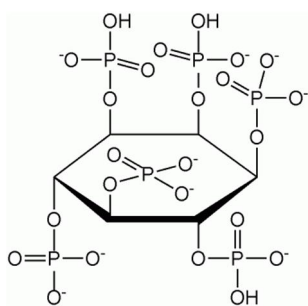
### **2.2.1. Natural sources**

#### ***Animal-based foods***

In foodstuffs containing muscle protein (meat, poultry, fish), P is bound to amino acid side chains, which are released during the digestion process as inorganic P (Massey 2003). P in animals occurs as nucleoproteins, phosphoproteins, phospholipids, and nucleotides and is also present in body fluids and some tissues (Molins 1991). The most important phosphoproteins found in foods are ovalbumin in eggs and casein in milk (Molins 1991). In milk, P exists in organic and inorganic forms (Gauchelli 2011). One-third occurs as inorganic P; 20% in esterbound casein amino acids, 40% in caseinate micelles, and the rest as water- and fat-soluble esters (Uribarri and Calvo 2003). The organic form is bound to organic molecules such as casein, phospholipids, DNA, RNA, nucleocides, nucleotides, and sugar Pi's (Gauchelli 2011). Inorganic P exists as Pi ions, and the distribution between different Pi forms depends on pH (Gauchelli 2011). In processing of milk, part of Pi can be transferred to aqueous phase. Some of the Pi is lost to whey in cheese processing (Gaucheron 2011), which explains the differing Pi contents in different dairy products.

## ***Plant-based foods***

Phytic acid i.e. *myo*-inositol hexakisphosphoric acid ( $C_6H_{18}O_{24}P_6$ ) is a natural P compound in many plant-based foods (Figure 3). Phytic acid and its salts are commonly called phytates (Reddy and Sathe 2002). In grains and seeds most of the P is in the form of phytate, which stores inositol and P (Reddy 2002). However, small amounts of P in plant products occur as phospholipids (Molins 1991).



**Figure 3.** *Chemical structure of phytic acid.*

Grains and seeds contain reasonably high amounts of phytate. Legumes are defined as dicotyledonous seeds, thus, they also contain phytate. Nuts also have high amounts of phytate compared with tubers, fruits, leafy products and vegetables (Reddy et al. 2002). Among leafy products and vegetables, tomatoes and okra contain high amounts of phytate, but no phytate is detected in many fruits (Reddy et al. 2002). However, in cereals phytate constitutes the majority of TP; in wheat 60-80%, in barley 55-70%, in oat 49%, in rye 38-66%, in brown rice 74-81%, and in polished rice 51-61% of TP occurs in the form of phytate (Reddy 2002). Because phytate is present in the outer layers of grains, in whole-grain cereals phytate contents are greater than in raffinated products. Phytate constitutes 50-70% of TP in soybeans, 27-87% in lentils, 40-95% in chickpeas, 40-95% in broad beans, 57-82% in peanuts, 36-53% peas, and 95% in tofu (Reddy 2002). In some tubers and fruits phytate P accounts for 20-34% of TP (Reddy 2002).

Phytate can be degraded during processing of foodstuffs or in the intestine (Sandberg 2002); however, the capability of the human intestine to degrade phytate is limited (Iqbal et al. 1994). Phytase enzymes are responsible for the enzymatic degradation: they appear in plants, microbes, and some animal tissues (Sandberg and Andlid 2002). Non-enzymatic degradation of phytate occurs at a low pH or at high temperatures and pressure. Phytate is degraded to lower inositol phosphates (IP<sub>5</sub>, IP<sub>4</sub>, IP<sub>3</sub>, IP<sub>2</sub>, IP), and the same process forms bioavailable, inorganic Pi (Sandberg 2002). Phytate is thermostable, and thus, it does not degrade easily with cooking, autoclaving, roasting or with other traditional heat processing methods (Sathe and Venkatachalam 2002). Hydrolysis of phytate during fermentation and bread-making is affected by many variables such as type and freshness of flours, use of yeast, pH of dough, water content, possible fermentation, leavening time and temperature (Türk et al. 1996, Sathe and Venkatachalam 2002). The effects of soaking on phytate hydrolysis depend on the soaking time and temperature. Because phytate is water-soluble, removal of soaking water has an impact on the final phytate content of the product (Sathe and Venkatachalam 2002). Phytates may also chelate with other minerals and impair bioavailability of Ca, iron and zinc in the foodstuffs (Lopez et al. 2002).

### **2.2.2. Other sources**

Food additive phosphates (FAPs) are used for a number of reasons; they enable food products to achieve, for example, better texture, taste, emulsification, acidification, leavening, anti-caking, moisture binding, antimicrobial action, color stability, iron binding, buffering, and freeze-thaw stability (Uribarri and Calvo 2013). In lean meat products (processed beef, pork, and chicken), FAPs are used to maintain tenderness and moisture during cooking (Uribarri 2013). A mixture of water, salt and sodium phosphate can be added to spice up meat products and prevent their drying. FAPs are used in ham, bacon and cold cuts as anti-oxidizing agents, stabilizing agents, and agents to improve taste and color. Also frozen fish products can be rinsed with sodium phosphate solution prior to freezing to prevent loss of protein during the thawing process. In dry food mixes, e.g. soups, FAPs are used as anti-caking agents. Also some canned fish

products contain FAPs. Without FAPs, the shelf-life of many foodstuffs would be shorter (Uribarri 2007). Further, it should be noted that some Ca-enriched products (e.g. soy drinks, other milk alternatives, and fortified juices) contain Pi in the form of CaPi (Fitzpatrick and Heaney 2003, Kung 2010). Moreover, some bakery products (cookies, biscuits, cakes, muffins) contain FAPs originated from baking powder. Phosphoric acid is used as an acidity regulator in cola beverages, and some countries also in other beverages. Table 4 displays approved FAPs, their E codes, and examples of products containing specific additives.

In the European Union, the use of FAPs is regulated by a Commission Regulation (European Union 2011). Legally allowed maximum amounts of FAPs are calculated as phosphorus pentoxide ( $P_2O_5$ ). One mg of P corresponds to 2.29 mg  $P_2O_5$ . The maximum amount allowed varies with the regulatory classification of the foodstuff; starch Pi's are usually distinguished from the others, and their addition to products is often allowed *quantum satis*. For breakfast sausages and processed meat, the maximum amount of FAP is 5000 mg/kg ( $P_2O_5$ ). For processed cheeses the maximum amount is 20 000 mg/kg ( $P_2O_5$ ), while for unripened cheeses the amount is 2000 mg/kg ( $P_2O_5$ ). For breakfast cereals, spreadable fats, and frozen potatoes, the maximum is 5000 mg/kg ( $P_2O_5$ ). For many foods, e.g. baby foods, FAP addition is not allowed (European Union 2011).

A market survey done in Finland (Suurseppä et al. 2001) showed that approximately 10% of food products on the market contain FAPs. After Finland joined the European Union, the food additive contents of foodstuffs became significantly higher due to more generous allowances in European Union legislation. FAP-content in cold cuts and sausages was doubled and that in liver pates rose by 25%, even though the levels were within allowed limits (20% of the maximum, earlier 30%) (Blomberg and Penttilä 1999). The maximum FAP amounts in sausages were tripled relative to the situation of the earlier legislation. However, the FAP levels in the analyzed products were far below the maximum limits, even though contents in processed cheeses were fairly high (Suurseppä et al. 2001). When comparing the P content based of analysis with that provided by manufacturers, about half of P in sausages seemed to be

**Table 4.***Phosphorus-containing food additives and their E codes (EVIRA 2011).*

<b>Additive</b>	<b>E code</b>	<b>Examples of products</b>
<b>Antioxidizing agents</b>		
<b><i>Orthophosphates</i></b>		
Phosphoric acid	E338	cola beverages
Monosodium phosphate	E339	puddings, cookies,
Disodium phosphate	E339	sweet pies
Trisodium phosphate	E339	
Monopotassium phosphate	E340	cheese, ice cream,
Dipotassium phosphate	E340	breakfast cereals
Tripotassium phosphate	E340	
Monocalcium phosphate	E341	enriched soya drinks,
Dicalcium phosphate	E341	salad dressings
Tricalcium phosphate	E341	
<b><i>Magnesium phosphates</i></b>		
Monomagnesium phosphate	E343	cheese, ice cream,
Dimagnesium phosphate	E343	dry food mixes
<b><u>Emulsifiers, stabilizers, thickening agents</u></b>		
<b><i>Diphosphates i.e. pyrophosphates</i></b>		
Disodium phosphate	E450	meat products
Trisodium phosphate	E450	(sausages, frankfurters),
Tetrasodium phosphate	E450	processed cheeses, sausages,
Tetrapotassium phosphate	E450	marinated meats,
Dicalcium phosphate	E450	products containing
Calcium di hydrogen phosphate	E450	baking powder
<b><i>Triphosphates</i></b>		
Pentasodium phosphate	E451	sausages, frankfurters,
Pentapotassium phosphate	E451	meatballs
<b><i>Polyphosphates</i></b>		
Sodium polyphosphate	E452	processed cheeses, sausages,
Potassium polyphosphate	E452	frankfurters, meatballs
Sodium calcium polyphosphate	E452	
Calcium polyphosphate	E452	
<b><i>Starch phosphates</i></b>		
Mono- starch phosphate	E1410	cold cuts, barbeque sauces,
Di- starch phosphate	E1012	ready to eat pizzas,
Phosphated di- starch phosphate	E1013	hamburgers
Acetylated di- starch phosphate	E1414	
Hydroxy propyl di- starch phosphate	E1442	
<b><u>Anti-caking agents</u></b>		
Sodium aluminium phosphate (sour)	E541	meat products, cup soups

originating from FAPs (Suurseppä et al. 2001). However, in the United States the use of FAPs is more common than in Finland. According to León et al. (2013), 44% of the 2394 best-selling branded grocery products contained FAPs; the most common were frozen foods (72%), dry food mixes (70%), packaged meat (65%), bakery goods (57%), soups (54%) and yogurts (50%). In their study, these products were matched to similar FAP-free products and P contents were analyzed. FAP-containing products had significantly higher P content than regular products. Authors also collected meals based on FAP-containing foodstuffs and regular foodstuffs, and they noticed that the difference between the mean P intakes was as high as the recommended daily allowance for P intake (736 mg/d) when consuming FAP-containing products. Carrigan et al. (2014) compared low-FAP and high-FAP meals based on 4-day menus developed by a specific nutritional software, and found that the intake of P from additives may reach harmful levels at least in kidney patients. Based on earlier data in the United States, the estimations of FAP intake have been criticized as being too low (Carrigan et al. 2014). The current use of FAP-containing products in Finland is unknown. The only study investigating FAP contents of foodstuffs in Finland was published in 2001 (Suurseppä et al. 2001). Since then, the market shares of products have changed, new products have become available, and food consumption patterns have changed (Mäkelä et al. 2008).

Also some dietary supplements may contain P (EFSA 2005). In a British study of multivitamin supplements, the P contents in supplements ranged between 15 to 1100 mg/portion (EFSA 2005). The contribution of these supplements to TP intake is, however, quite low at the population level. The NHANES 2007-2008 data in the United States showed that average P intake via supplements was 108 mg/day/portion (range 31-130 mg/d) (Uribarri 2013). To date, P contents of dietary supplements in Finland have not been analyzed.

Recently, concern has arisen on P content of the inactive ingredients in both prescription and over-the-counter medications (Uribarri 2013). According to Uribarri, these medications can cause a rise in TP intake if many P-containing medications are used at the same time, and Uribarri notes that in the management of CKD this can cause problems when estimating the TP intake on P-restricted diets. According to Calvo and Park (1996), also packaging material



and other P-containing compounds used in the manufacturing process may end up in foodstuffs, but they consider the effect on TP intake to be negligible.

#### **2.2.4. Methods for assessment phosphorus contents and bioavailability in foodstuffs**

##### **2.2.4.1. Basic chemical methods to analyze phosphorus contents**

Different chemical methods can be used to analyze the P content in foodstuffs. However, because foods have complex textures and P can be bound to other elements, the same methods as used for pure  $P_i$  solutions cannot be used. The samples are usually pre-treated, e.g. the fat is removed, before the analysis. In colorimetric methods, the P content in foodstuffs is assessed based on the intensity of the color formed in the reaction. The color can be assessed by either a method based on formation of ammonium phosphomolybdate, which is reduced to ammonium blue (Olson and Summers 1982, Nordic Committee on Food Analysis 1994), or a molybdate vanadate method, where P reacts with molybdate vanadate complex and forms a yellow color (Olson and Summers 1982, AOAC 965.17, Phosphorus in animal feed and pet food). This method is not as precise as the former one, but it can be used for the assessment of higher P contents. Most of the P contents in foodstuffs in the Finnish food composition database Fineli<sup>®</sup>, especially cereals, are analyzed using this method (Varo 1980). In titrimetric methods (AOAC Official method 964.06 Phosphorus in Animal Feed, AOAC Official Method 948.09 Phosphorus in flour), the added phenoliphtalein forms a pink color in the titrated solution. Molybdate solution and Na hydroxide can be used as a reagent solution in the first phase, and either Mg nitrate or molybdate solution in the second phase.

P contents can also be assessed by plasma-atomic emission spectrometry, but not by atomic absorption spectrometry or atomic fluorescence spectrometry (Lajunen 1986). In this method, the high temperature produces kinetic energy for the particles, which transforms the atoms to an excited stage and ions to a higher level, where their excess energy is emitted as photons. Each compound

emits its own radiation with many wave-lengths. The emission forms a spectrum, the compounds of which can be recognized and the contents analyzed. Inductively coupled plasma mass spectrometer (ICP-MS) is one of these plasma emission spectrometry methods. In ICP-MS, the recognition and sorting of ions is based on their mass-charge ratio. The device is sensitive and the repeatability of the measurements is good (PerkinElmerSciex Instruments 2001). Also inductively coupled plasma optical emission spectrometry (ICP-OES) and inductively coupled plasma atomic emission spectrometry (ICP-AES) can be used to analyze P contents in foodstuffs; with these techniques, the intensity of the emission describes the concentration of the element in the sample.

#### **2.2.4.2. Methods to assess mineral bioavailability**

##### ***In vivo* methods**

Methods for assessment of mineral bioavailability *in vivo* utilize living organisms such as animals, humans and plants (Hur et al. 2011). These methods may, for example, investigate mineral contents in serum or urine, mineral balance in the presence of stable or radioactive compounds, or effects of the mineral on target organs (Heaney 2001). When evaluating only serum concentrations, the strictly regulated metabolism may not result in dose-dependent changes in concentrations, and moreover, for many nutrients gold standards are not available as biomarkers of bioavailability. When analyzing only urine excretion, it is important to take into account the glomerulus filtration rate (GFR), which depends on the subject and causes biological changes in the results. Investigating the impact on target organs is effective, but biological responses differ between subjects. Radioactive methods are sensitive, quick, and easy. A labeled isotope, stable or radioactive, can be used to determine the amount of absorbed mineral. Also ileostomic patients have been used as subjects to investigate mineral bioavailability because their intestinal contents can easily be accessed (Kivistö et al. 1986, Sandberg et al. 1987, Sandberg et al. 1988). It is notable that in ileostomic patients only absorption in the area of small intestine can be evaluated, not possible absorption in the colon

(Kivistö et al. 1986). Generally, human studies are time-consuming, expensive and difficult to carry out (Sandberg 2005).

Using animal studies to investigate mineral bioavailability also has some drawback due to differences between animals and humans in mineral needs and absorption. For example, Fe absorption in rats is dissimilar to that in humans, thus hindering extrapolation of results to humans (Sandberg 2005). Rats have phytase activity in their gut, while humans have only marginal phytase activity (Hotz 2005), affecting the results when studying bioavailability of plant-based foodstuffs.

### ***In vitro* methods**

Cells or biological molecules can also be studied outside their normal biological context. *In vitro* methods are faster, less expensive, and easier to control than *in vivo* methods (Sandberg 2005). *In vitro* methods usually mimic the processing of food in the alimentary tract. In *in vitro* methods evaluating mineral bioavailability, the foodstuff is first digested enzymatically (e.g. by alpha-amylase, pepsin and pancreatine) and the rest of the processing depends on the filtration method chosen. In *in vitro* dialysis/solubility methods, the digestion is two-phased: first the processing of food in the alimentary tract is mimicked and then the sample is dialyzed through a half-permeable membrane based on molecular weight describing the absorption process (Sandberg 2005). Depending on the method, the incubation times, enzymes used, and end processing are variable (Hur et al. 2011). Restrictions of *in vitro* methods are that the interaction between the alimentary tract and food or food components cannot be measured (Sandberg 2005). In practice, the transit time in the alimentary tract depends on the content of the meal, and absorption capability of the different parts of the alimentary tract differs. The content and concentrations of alimentary enzymes also depend on the content of the meal. Neither intestinal microbiota nor circulation exist in *in vitro* methods.

## **2.3. Gaps in the knowledge on intake and bioavailability of dietary phosphorus**

### **2.3.1. Food additive phosphorus intake**

When assessing the P contents in foodstuffs, the natural P and FAP cannot be distinguished analytically (Suurseppä et al. 2001), and thus, P contents in food composition databases are reported as total P. Moreover, in food composition databases, P contents of the recipe-based foodstuffs are calculated based on nutrient contents of raw materials, and not on chemically analyzed values (National Institute for Health and Welfare 2011). Thus, the amounts of FAPs may have not been taken into account. The consumption of ready-to-eat meals has increased four-fold over the last two decades in Finland (Mäkelä et al. 2008), and food composition databases have not taken into account possible FAPs in these foodstuffs (Oenning et al. 1988, Sullivan et al. 2007). Thus, often the only way to obtain information about FAP contents is from the manufacturers, which can be problematic (Calvo and Park 1996). Voluntary labeling of P contents in foodstuffs has not been popular in the United States, even though the National Kidney Association recommends labeling (Uribarri and Calvo 2013). However, in the European Union, the legislation requires that any food additives used are listed as E codes or their names on the package (European Union 2011).

In the United States, the estimated FAP intake in 1979 was 320 mg/day, approximately 20-30 % of total P intake (Greger and Krystofiak 1982). The P intake from additives in the United States has been estimated to have increased from 470 mg/d in 1990 to 1000 mg/d in 2012 – now with 50% of P intake coming from additives as “hidden phosphorus” (Winger et al. 2012). Information about the use of FAPs in Finland is scarce and out of date. Finnish Food Authority and Customs Laboratory conducted a survey of FAP-containing foodstuffs in 2000. Based on their results, the main sources of FAPs were sausages and other meat products, baked goods leavened with baking powder, and some ready-to-eat and processed meals such as pizza (Suurseppä et al. 2001). Also phosphoric acid-containing cola beverages were considered a

potential high-FAP source. The authors concluded that FAP intake can be excessive in small children or persons consuming particular products, e.g. sausages and other processed meat products or cola beverages in abundance. An earlier survey showed that mean FAP intake from meat products was 13 mg/d among women and 38 mg/d among men (Ovaskainen et al. 2000). The mean intake among adults was 25 mg/d. In this survey, only FAP intake from meat products was estimated; food consumption data was based on in the National FINDIET Study 1997, and analytical values were derived from Blomberg and Penttilä's surveys (1999). However, the FAP contents in the products were clearly lower than the allowed maximum amounts in foodstuffs (Blomberg and Penttilä 1999). Further, the current FAP intake in Finland is not known due to lack of comprehensive studies.

Oenning et al. (1988) compared P and Ca intake based on chemically analyzed results and nutrient databases. On the basis of database values, the P intake was 15-25% lower than when it was analyzed chemically, although the correlation between the methods in Ca intake was good. This may cause underestimation of the true P intake. Most studies comparing P contents of FAP-enhanced and regular products are conducted on animal products (Sherman and Mehta 2009a, Sherman and Mehta 2009b, Benini et al. 2011). Sullivan et al. (2007) did a survey about chicken products: 35 of the analyzed 38 chicken products contained FAPs (92%). In almost all FAP-containing products, the P contents were higher than content expected from nutrient databases. P contents between manufacturers differed by up to 100 mg/100 g product. Researchers concluded that additives seemingly increased P content of chicken products. Recently, Carrigan et al. (2014) analyzed P contents of high- and low-FAP-containing meals over an entire day, and surprisingly, no significant differences were found between calculated and analyzed P content of high-FAP-containing meals, contradicting earlier results. Nevertheless, in examining the low-FAP-containing meals, the P content between calculated and analyzed content did show a difference. Calvo and Tucker (2013) propose as an important future work to improve food composition databases that the natural and additive sources of P be identified.

### **2.3.2. Bioavailability of phosphorus**

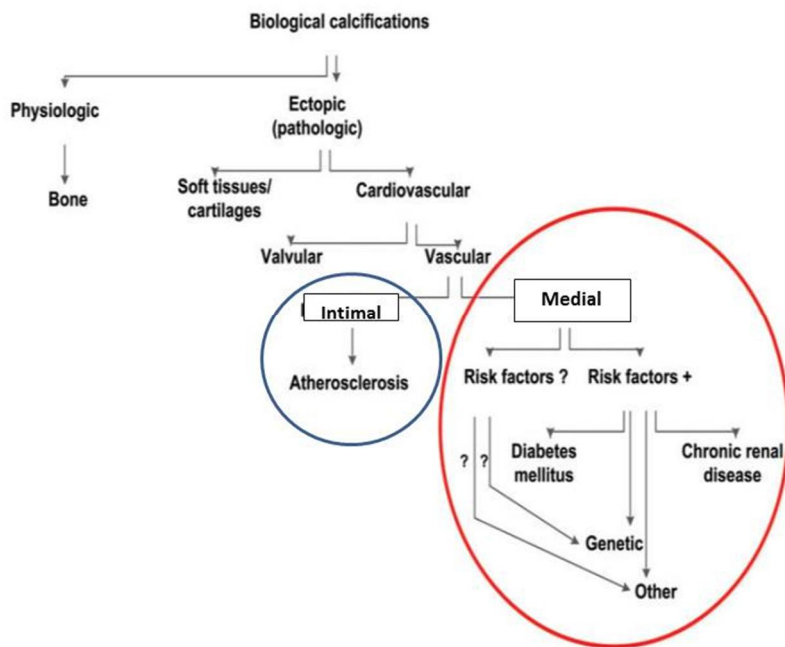
FAP-containing foodstuffs have been shown to cause more adverse effects in Ca and bone metabolism than foods without FAPs (Bell et al. 1977, Kemi et al. 2006, Karp et al. 2007, Karp et al. 2012) indicating better bioavailability of FAPs, however, in above-mentioned studies only in terms of mineral metabolism markers. No differences have emerged between different forms of FAPs, e.g. mono- and polyphosphates, concerning mineral metabolism (Karp et al. 2013). Also the bioavailability of P in animal origin foodstuffs has been stated to be better than in vegetable origin foodstuffs (Moe et al. 2011, Karp et al. 2012). In the whole-foods approach intervention study of Karp et al. (2007), the metabolic response was different for different foods (cheese, meat, whole-grain), indicating different bioavailability. However, no generally reliable methods to assess bioavailability of P in the foodstuffs have been developed. Benini et al. (2011) and Cupisti et al. (2012) have developed methods to assess different fractions of soluble P in meat products with or without FAPs, however, these methods need further development to be used in P analysis of other foodstuffs.

Further, the recent study of Chang et al. (2014a) instigated discussion about the assessment of P bioavailability (McCarty 2014, Chang et al. 2014b). McCarty paid attention to the lower bioavailability of plant-derived P and proposed use of a correction factor in P intake for plant-derived P. However, according to Chang et al., correction factors are likely to introduce error into results until reliable methods are developed to estimate P bioavailability. The authors further stated that problems are caused by unknown exposure to FAPs as well as by food preparation. They recommended the use of 24-h urine Pi excretion measurement, which has been shown to be a reliable method for estimating true dietary P intake (Morimoto et al. 2014). However, 24-h urine collections have not been available in many studies, including that of Chang et al. Calvo and Tucker (2013) propose the use of algorithms to better quantify P bioavailability, thus producing better estimates for actual P intake. Thus, in addition to these, there is a need to develop a method to analyze bioavailable P contents in foodstuffs.

## 2.4. Phosphorus, vascular calcification and cardiovascular diseases

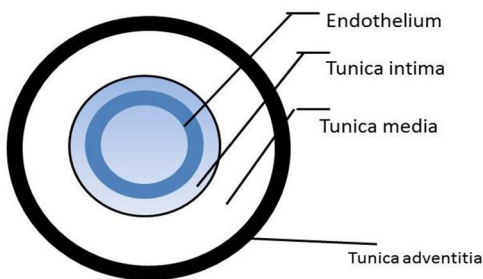
### 2.4.1. Calcification and cardiovascular diseases

Mineralization occurs in normal bone, but when it occurs in soft tissue and not as a result of aging, it is called ectopic calcification. In the vascular system, this is a pathological condition due to mineral (mostly Ca and Pi) deposition in the vascular vessel wall (Marulanda et al. 2014). Vascular calcification is associated with atherosclerosis, diabetes, kidney disease, and some genetic and metabolic disorders (Wu et al. 2013). It predicts independently cardiovascular events in the normal population, renal patients, and diabetics (Marulanda et al. 2014). Figure 4 presents the nomenclature of biological calcifications.



**Figure 4.** *Nomenclature of biological calcifications (adapted from Lanzer et al. 2014).*

Vascular calcification comprises two distinct disorders: intimal (atherosclerotic) and medial calcification (Mönckebergs sclerosis) (Lanzer et al. 2014). Knowledge about molecular mechanisms in vascular calcification is growing. Deposition of CaPi i.e. hydroxyapatite, different initiating and propagating molecular mechanisms, and diverse crystalline compositions of Ca apatite crystals can be present in vascular calcification, in both intimal and medial calcification (Lanzer et al. 2014). Both of these calcifications result in ectopic vascular calcification, but the triggering biochemical cascades between intimal and medial calcification differ; in intimal calcification atheroplaque is formed, but in medial calcification the cascades are initiated by damages to elastic fibers (Lanzer et al. 2014). Figure 5 presents a cross-section of a normal vessel. Normally, the intimal layer consists of endothelial cells and a small amount of connective tissue. In the case of atherosclerosis, inflammation, thickening, and calcification occur. Calcification seems to increase atheroma plaque rupture risk, mainly in older persons with heavily calcified arteries (Sakakura et al. 2013). Further, media consists of smooth muscle cells and an elastin-rich extracellular matrix, and medial calcification occurs in the elastic lamina. Medial calcification, affecting diabetics, renal patients, persons with osteoporosis, and hypertension patients, leads to stiffening of the arterial wall, is associated with elevated blood pressure, and entails a higher risk of cardiovascular mortality (Wu et al. 2013).



**Figure 5.** *Cross-section of a vessel.*

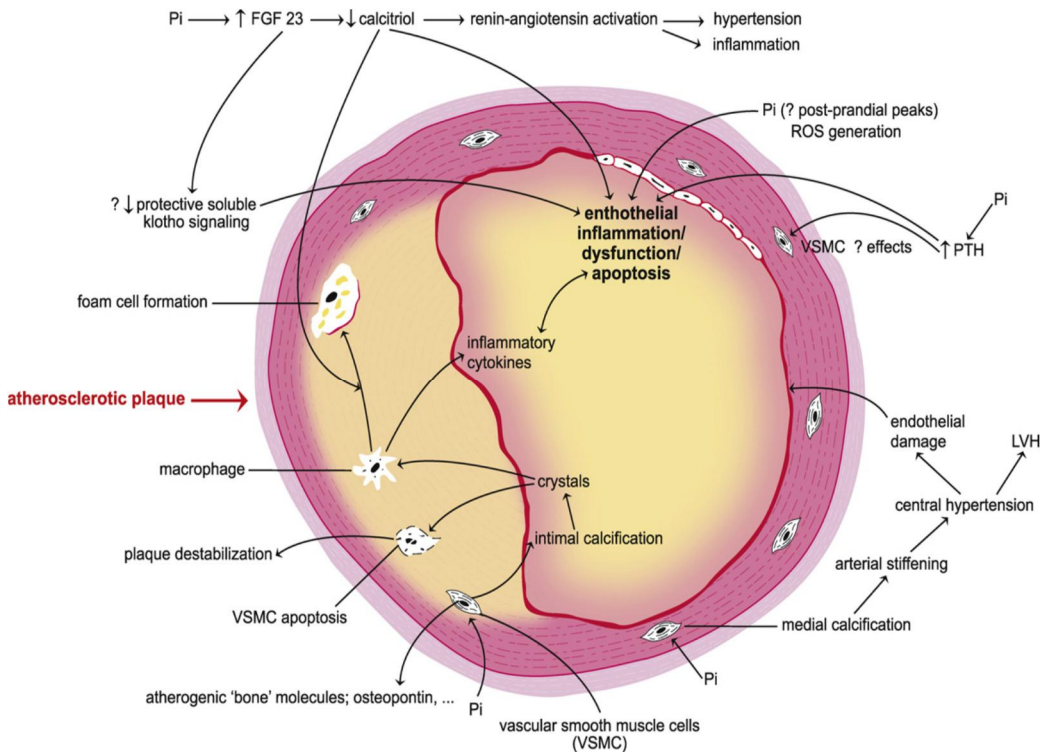


Vascular calcification was earlier considered a passive phenomenon occurring within aging, but now it is known to be an active process with inductive and inhibitive reactions (Wu et al. 2013). The process of calcification is similar to bone formation; the same mediators participate in both processes, and in calcification vascular cells undergo a phenotypic change to osteoblast-like cells. The major mechanisms revealed thus far for calcification are disturbed anticalcific processes, induction of osteochondrogenesis, cell death, abnormalities in Ca and Pi homeostasis, formation and circulation of calciprotein particles, and modifications in the matrix (Wu et al. 2013). Calcified vascular smooth muscle cells express the same proteins as osteoblasts (Jono et al. 2000), and it has been suggested that the regulators of bone metabolism, osteoprotegerin/RANK/RANK ligand axis, bone morphogen protein, matrix Gla protein and fetuin A, may play a role in vascular calcification, indicating mineralization similar to bone (van Campenhout and Golledge 2009, Figueiredo et al. 2013). Further, CVD and osteoporosis have been found to be associated in epidemiological studies (see den Uyl et al. 2011), giving rise to the hypothesis that these two diseases may share some common mechanisms as well as P's common role in them. Data from some epidemiological studies indicates associations between vascular calcification, impaired bone metabolism, and increased mortality (Cannata-Andia et al. 2011). In renal patients these associations are even stronger.

Pi can participate in vascular calcification via multiple mechanisms (see Figure 6); for instance, cytotoxicity of Pi in cultivated vascular cells, Pi acting as an endothelial toxin, and calciprotein particle production have been suggested to underlie P's role in CVD. Pi may act as an endothelial toxin because in the presence of high Pi concentrations endothelial cells have been shown to impair nitric oxide synthesis, increase reactive oxygen species generation, and undergo apoptosis. Calciprotein particles induce phenotypical changes in vascular cells, but it is notable that Pi is not the only factor that participates in crystal formation and dissolution under conditions of high intracellular Ca level (Ellam and Chico 2012).

Elevated S-Pi concentrations also provoke metabolic responses; increases in FGF23 and PTH concentration, and decreases in calcitriol concentration, which

may also underlie P's role in vascular health. FGF23 can directly act on cardiomyocytes to promote left ventricular hypertrophy, which is also linked to low vitamin D status and elevated S-PTH concentrations. Calcitriol can act in the renin-angiotensin system by suppressing renal renin secretion; increased renin concentration in blood has been shown to be a CVD risk factor. Calcitriol can also interfere in CVD via anti-inflammatory effects on vascular macrophages (McCarty and DiNicolantonio 2014).



**Figure 6.** *Potential mechanisms by which phosphate could be involved in vascular calcification (from Ellam and Chico 2012). Pi phosphate; FGF23 fibroblast growth factor 23; ROS reactive oxygen species; VSMC vascular smooth muscle cells; PTH parathyroid hormone; LVH left ventricular hypertrophy*

#### **2.4.2. Methods to measure calcification and risk or progression of atherosclerosis**

##### ***Biomarkers of cardiovascular disease risk***

Several biomarkers can be used to measure CVD risk. Traditional risk factors, such as high concentrations of low-density lipoprotein cholesterol (LDL-C) and high blood pressure are well known, but other factors have also been considered. Inflammation markers measured in blood, such as white blood cells, C-reactive protein (CRP), interleukin-6, and macrophage colony-stimulating factor are connected to atherogenesis and plaque instability (Ikonomidis et al. 2008). Recently, markers of mineral metabolism, such as osteocalcin, osteopontin, bone morphogen protein, fetuin A, matrix Gla protein, and FGF23, have been proposed to act as potential signaling molecules in arterial calcification (Everett 2010). The strongest evidence exists for associations between CVD and low fetuin A, low undercarboxylated matrix Gla protein, and high FGF23 concentrations (Everett 2010). Nevertheless, more research about these markers and prediction of CVD risk is required.

##### ***Intima-media thickness measurement and other non-invasive methods to measure vascular calcification***

Various non-invasive measurement methods can be used to assess vascular calcification: arterial stiffness (a predictor of mortality and coronary heart disease) with pulse wave analysis or pulse wave velocity, endothelial function assessment with flow-mediated dilation, nitro-glycerin-mediated dilation and brachial artery diameter measurement, peripheral flow with ankle-brachial index either at rest or after exercise, and arterial wall thickness measurement in terms of carotid intima-media thickness (cIMT) (Holewijn et al. 2010). Coronary artery calcification can be measured by computed tomographic angiocardiology (Kwan et al. 2013). However, cIMT measurement is the most common and easiest method to use. cIMT measurement can be applied to assess CVD risk and to monitor disease progression (O'Leary and Bots 2010). Ultrasound imaging of the carotid gives reliable prediction of state of

atherosclerosis in other arteries, and the results correlate with histology of the vessel. Many epidemiological studies have established cIMT as a marker of subclinical atherosclerosis, and it is associated with CVD risk factors and with prevalent and incident CVD (O'Leary and Bots 2010). Echoimaging of the carotid has revealed that IMT correlates even in young persons with future CVD risk (Raitakari et al. 2003).

## **2.5. Current knowledge on phosphorus and cardiovascular health from an epidemiological point of view**

It has long been known that renal patients have impaired mineral metabolism and are unable to excrete P properly, causing e.g. vascular calcification if not treated with P-restricted diets (Qunibi et al. 2002). Information from short-term and intervention studies in the general population suggests that high P intake especially together with low Ca intake might be harmful for bone and Ca metabolism (Calvo and Park 1996, Kärkkäinen and Lamberg-Allardt 1996, Kemi et al. 2006, 2009, 2010). Also different P sources have different effects on mineral metabolism: FAP seems to be more harmful than natural P (Karp et al. 2007, Kemi et al. 2009).

In recent studies, some concern has been raised about excess dietary P intake and vascular calcification, cancer, obesity, and hypertension (Anderson 2013). Several organs and tissues are affected by elevated S-Pi concentrations following high P intake (Anderson 2013). High S-Pi concentrations have also been speculated to cause inflammation, adipocyte hyperplasia, increased triglyceride concentrations, insulin resistance, tumors, and Alzheimer's disease, although data for neurological conditions are limited (Anderson 2013). Excessive phosphorylation of protein kinase Akt in all kinds of cells has been suggested to be the main actor in cell metabolism associated with many chronic diseases (e.g. cancer, type 2 diabetes, and neurological and cardiovascular diseases) (Hers et al. 2011).

### **2.5.1. Serum phosphate and cardiovascular health**

Decreased P excretion followed by hyperphosphatemia is associated with progression of secondary hyperparathyroidism, renal osteodystrophy and increased mortality and morbidity in dialysis patients (Slatopolsky and Delmez 1994, Block et al. 1998, Block et al. 2004). Hyperphosphatemia is also linked to vascular calcification and CVD mortality in these patients (Block et al. 1998, Qunibi et al. 2002, Kestenbaum et al. 2005). However, in the last decade, high

S-Pi has been found to be associated with CVD risk also in large population-based studies of subjects with normal renal function (Tonelli et al. 2005, Dhingra et al. 2007, Foley et al. 2008). Table 5 presents the main findings of studies on S-Pi and cardiovascular health in the general population. Elevated S-Pi even in young persons was associated with atherosclerosis risk (Foley et al. 2009) and higher cIMT, a subclinical marker of atherosclerosis (Ruan et al. 2010). In an earlier study of Onufrak et al. (2008), S-Pi was associated with cIMT in males but not in females. A Korean study (Park et al. 2010) reported an association between lower S-Pi and less coronary artery calcification. In the MESA study in individuals with normal kidney function to moderate kidney disease, high S-Pi was associated with high ankle brachial pressure, a marker of arterial stiffness, however, not with arterial elasticity (Ix et al. 2009). Further, Kendrick et al. (2010) showed in the NHANES population that S-Pi, even within normal reference limits, was associated with higher ankle brachial pressure. In a longitudinal study, S-Pi independently predicted coronary artery calcification (Tuttle and Short 2009). Larsson et al. (2010) reported that elevated S-Pi was associated with risks of total, cardiovascular, and non-cardiovascular mortality in the general population. High S-Pi was related to greater risk of mortality also in the study of Sim et al. (2013).

The reported data on the deleterious effects of high S-Pi is from follow-up and cross-sectional studies, but the only case-control study (Taylor et al. 2011) failed to show evidence of an association between plasma Pi (P-Pi) and development of coronary heart disease. However, the subjects in the latter study comprised only males. The only meta-analysis on the topic, including 24 studies, concluded that S-Pi non-linearly predict risk of CVD deaths/events and total mortality, but not coronary heart disease events (Li et al. 2014). Nevertheless, these data cannot be extrapolated to the general population because also studies carried out on subjects with impaired kidney function (CKD stages 1-2 [range 1-5]) and CVD were included.

Thus, results of these epidemiological studies support the evidence that high or elevated-normal S-Pi concentrations may be harmful for vascular system and health. Nevertheless, some studies show differences between the sexes in the association between S-Pi concentrations and CVD risk factors. Some authors

have stated that S-Pi could be considered a novel cholesterol, and in the future Pi binders (now used in CKD patients) could possibly be used also in the normal population in the prevention of CVD (Ketteler et al. 2013, McCarty and DiNicolantonio 2014).

### **2.5.2. Dietary phosphorus and cardiovascular health**

Several studies on the effects of high dietary P intake have been conducted with uremic animal models that better describe circumstances under deficient kidney functions (e.g. El-Abbadi et al. 2009, Nikolov et al. 2012, Che et al. 2013). However, a high-P diet has increased vascular calcification in mice (Román-García et al. 2010), and in ApoE-deficient mice reduced atherosclerosis formation in an atherosclerotic condition, which was an unexpected result (Shiota et al. 2011). Dietary P restriction also reduced vulnerability to high-cholesterol diet-induced dyslipidemia in mice (Tanaka et al. 2013).

Only limited data are available on the effects of a high-P diet on cardiovascular health outside CKD in humans (see Table 6). Acute high P intake (1200 mg) reduced endothelium-dependent flow-mediated brachial artery dilation in eleven healthy men (Shuto et al. 2009). In the study of Yamamoto et al. (2013) a high dietary P intake (assessed by food frequency questionnaires [FFQs]) in subjects (n=4494, mean age 62 years) was associated with left ventricular hypertrophy among women (highest P intake quintile 1346-4069 mg/d) but not among men. Their data was adjusted for demographics, Na and energy intake, lifestyle factors, comorbidities, and established left ventricular hypertrophy risk factors. Chang et al. (2014a) showed in the NHANES cohort (n=2686, age 20-80 years) that dietary P intake >1400 mg/d and P density of the diet >0.35 mg/kcal were associated with all-cause mortality. High P density (>0.35 mg/kcal) was associated with CVD mortality, but no such results were found for total dietary P intake. Interestingly, also low P density (<0.35 mg/kcal) was associated with increased mortality risk. In that study, P intake was assessed by 24-hour recalls, and the data was adjusted for demographics, CVD risk factors, kidney function, and energy intake, and the results remained so when Na and saturated fat intake were introduced as covariates. Neither differences between

diet quality subgroups based on the Healthy Eating Index were perceived. In a recent Korean study on subjects with preserved renal function, no association between dietary P intake and coronary artery calcification was found (Kwak et al. 2014), but the mean P intakes in the study population were very low, less than 800 mg/d. However, because P and Ca interact (Peacock 2010), it is important to consider intake of Ca as a confounding factor. These studies failed to do this and also the sources of dietary P intake were not separated, which may explain the finding that no association between dietary intake of P and coronary artery calcification.

Some cross-sectional population-based studies propose that high dietary P intake is associated with lower blood pressure (Joffres et al. 1987, Beydoun et al. 2008, Elliott et al. 2010). However, the effects of confounding factors cannot be excluded in these studies. In the study by Elliott et al., the study population was from four different countries with differing main P sources and total P intakes were not reported. Other limits of their study are high intercorrelations between P and Ca intake and between P and Mg intake. Also Joffres et al. (1987) reported high intercorrelations between the nutrients, and in the multivariate analyses the data were adjusted only for age and body mass index (BMI). In the study of Beydoun et al. (2008), in addition to P, cheese and low fat milk consumption were inversely associated with blood pressure, which may reflect more the role of dairy products than P itself. Similarly, Alonso et al. (2010) showed in their prospective follow-up study on the MESA and ARIC study populations (n=13 444, P intake measured by FFQ) that P from dairy products, but not from other sources was associated with lower baseline blood pressure. The mean P intakes in the lowest quintiles were about 900-1000 mg/d, compared with 1500 mg/d in the highest intake quintiles, within the overall reported P intake range from 0 mg/d to 3570 mg/d. However, the intakes of 0mg/d can be questioned; that low intakes do not appear real, and those subjects could have been excluded from the analyses. However, according to the authors, dairy products (with or without the effects of P) may mainly explain the negative association between P and blood pressure. When other dietary factors (e.g. intakes of Ca, Mg, potassium) were introduced to the models, the effects of dietary P on blood pressure were attenuated, but remained statistically significant when all subjects were pooled in the analyses. Further, based on



**Table 5.** Evidence of high serum phosphate (S-Pi) concentrations and cardiovascular disease risk in the general population.

Reference	n	Age (years)	Study design	Follow-up time (years)	High or high-normal S-Pi	Outcome/association with high S-Pi
<i>Case-control studies</i>						
Taylor et al. 2011	1259	mean 64	nested case-control follow-up	10	-	P-Pi not associated with development of CHD
<i>Follow-up studies</i>						
Tonelli et al. 2005	4159*	21-75	post hoc analysis follow-up	5	high, most normal range	risk of death, CV events
Dhingra et al. 2007	3368	mean 44	prospective follow-up	16	high	risk of CVD
Foley et al. 2008	15732	mean 54	follow-up prospective	13	high	CVD risk factors and outcomes
Tuttle and Short 2009	883	>18	longitudinal observational follow-up	6	high	coronary artery calcification prediction
Foley et al. 2009	3015	mean 25	prospective observational cohort	15	higher in normal range	greater coronary artery calcification
Larsson et al. 2010	2176	mean 50	prospective cohort	30	high	total and CV mortality
Sim et al. 2013	94989	mean 50	retrospective longitudinal cohort	11	high	end stage renal disease and mortality risk
<i>Cross-sectional studies</i>						
Onufriak et al. 2008	13340	45-64	cross-sectional community cohort	-	high	cIMT males, not females
Ix et al. 2009	1370**	45-84	cross-sectional	-	high	high ankle brachial index***
Park et al. 2010	402	mean 51	cross-sectional	-	lower in normal range	low S-Pi: less coronary artery calcification
Kendrick et al. 2010	581	>40	cross-sectional	-	high in normal range	high ankle brachial pressure
Ruan et al. 2010	1210	24-44	cross-sectional	-	higher in normal range	cIMT

CHD coronary heart disease; CV cardiovascular; CVD cardiovascular disease; cIMT carotid intima-media thickness, P-Pi plasma phosphate

\*subjects had earlier myocardial infarction, \*\*of which 440 with moderate kidney disease, \*\*\* pulse pressure and arterial elasticity non-significant

**Table 6.** *Evidence on high dietary phosphorus (P) intake and cardiovascular disease-related problems in animals and humans.*

<b>Refence</b>	<b>Animal/human</b>	<b>Amount of P</b>	<b>outcome/association with high P intake</b>
Román-García et al. 2010	animal	high P diet	increased vascular calcification
Shiota et al. 2011	animal	high P diet	reduced atherosclerosis in ApoE-deficient mice
Tanaka et al. 2013	animal	P restriction	reduced diet-induced dyslipidemia in mice
Shuto et al. 2009	human	acute high P intake	reduced endothelial function of vessels
Yamamoto et al. 2013	human	high P intake	increased left ventricular hypertrophy, only women
Alonso et al. 2010	human	P from dairy, not other sources	lower baseline blood pressure and reduced risk of hypertension
Elliott et al. 2008	human	high P intake	inverse association with hypertension
Chang et al. 2013	human	high P intake	increased all-cause mortality

other studies, it is more likely that high P intake impair vascular function via acute high S-Pi and thereby increases blood pressure (Shuto et al. 2009, Lanzer et al. 2014).

Even though associations between both high S-Pi and dietary P intake and CVD health have been reported, no associations between both S-Pi and dietary P intake and CVD have been found in the same studies. Uribarri and Calvo (2014) state in their editorial in the American Journal of Clinical Nutrition that the lack of an association between dietary P and S-Pi (and CVD) is due to failure to take into account several confounding factors. They propose that S-Pi is not a proper indicator of dietary P intake, and the association between high S-Pi and CVD might be due to some other reason. This topic is now under active discussion, and according to a recent opinion in the European Heart Journal, not only high S-Pi but also high dietary P intake has been proposed a potential CVD risk factor (Ketteler et al. 2013).

### **2.5.3. Regulators of phosphate metabolism and cardiovascular health**

Ellam and Chico (2012) proposed that Pi regulatory hormones may respond better to dietary P load than only S-Pi. It has been suggested that FGF23 or PTH or other regulators of P metabolism could be the main reasons for the detrimental effects of P (Uribarri and Calvo 2014). PTH and FGF23 are both known to increase with high dietary P intake, FGF23 in a prolonged way, and PTH also acutely, and persistently with continuous exposure to high P intake (Block et al. 2013). PTH and FGF23 suppress the active metabolite of vitamin D, 1,25(OH)<sub>2</sub>D, and all of these mediators have been associated independently with adverse health effects even within normal S-Pi concentrations in healthy subjects (Block et al. 2013). In renal patients, many studies have shown that elevated FGF23 was associated with CVD or mortality (e.g. Kendrick et al. 2011, Isakova et al. 2011). In patients with prevalent CVD, higher FGF23 was independently associated with mortality (Parker et al. 2010), and Ix et al. (2012) found that high FGF23 was associated with increased all-cause mortality and heart failure incidence also in community-dwelling individuals without CKD, although the association was stronger in CKD patients (Ix et al. 2012). FGF23

has also been associated with left ventricular hypertrophy in longitudinal study in older adults (Mirza et al. 2009). However, in the prospective case-control study of Taylor et al. (2011) plasma FGF23 was not associated with incident coronary heart disease among males.

Moreover, a meta-analysis of 15 prospective studies showed that high PTH was associated with increased risk of CVD events (van Ballegooijen et al. 2013). However, for total CVD events (10 studies included in the analysis) only three of the studies showed a positive association between PTH and CVD, with the rest seven studies showing no association (van Ballegooijen et al. 2013). Nevertheless, in a longitudinal study in older adults not included in the meta-analysis, high PTH even within normal range, was associated with CVD (Buizert et al. 2013). In the same study, high PTH was associated with abdominal aortic calcification among males, but not among females (Buizert et al. 2013).

Even though low 25(OH)D status has been related to CVD, recent reviews state that the causal link between low 25(OH)D status and CVD remains to be elucidated (Lamberg-Allardt et al. 2013, Cashman 2014). From a mechanistic point of view, however, low concentrations of 1,25(OH)<sub>2</sub>D, the active form of vitamin D, has been shown to have a potential role in CVD (Cashman 2014). Thus far, no consensus exists about the role of regulators of P metabolism and their role in CVD.

### 3. Aims of the thesis

This thesis aims to offer new insights into the potential health risks related to high dietary P intake in Finland by providing knowledge on the bioavailability of dietary P, and on the association of high dietary P intake with cardiovascular disease (CVD) risk factors in the general population. The first aim was to investigate *in vitro* digestible and total phosphorus (DP and TP, respectively) contents in differently processed cereals and other selected plant-based foodstuffs using a new analysis method. Another aim was to determine at a public health level whether high dietary P intake, especially in the form of highly absorbable food additive phosphorus (FAP), is associated with vascular calcification in terms of high carotid intima-media thickness (cIMT), and thus, with increased risk of CVD, in a middle-aged Finnish population. Previous to this work no studies on these topics have been published. Specific research questions were as follows:

**I: Are there differences in *in vitro* DP and TP contents in differently processed cereals? Does the processing affect the DP concentrations? Is the analysis method of *in vitro* DP trustworthy?**

**II: Are there differences in *in vitro* DP and TP contents of selected commonly used plant-based foods and beverages? Do the FAP-containing foodstuffs have higher proportions of DP than FAP-free products?**

**III: Is high dietary P intake associated with higher cIMT in a general middle-aged Finnish population in a cross-sectional design? Are there differences in associations between total dietary P and FAP intake in cIMT?**

## 4. Subjects and methods

### 4.1. Food studies (Studies I and II)

#### 4.1.1. Samples

##### *Study I*

The samples were different cereal products commonly used in Finland (Table 7). The samples were bought at two local grocery stores in Helsinki in June 2007. The rye sourdough originated from a bakery in Helsinki. All flours, grits, yeasts, and breads were from the same batch to ensure homogeneity of the samples. Three different kinds of breads were baked: sourdough rye bread, sourdough wheat bread, and yeast-fermented (non-soured) wheat bread. Barley porridge was made with barley grits, water, and salt. More information about the home-baked samples is provided in Appendix (Table 1). All breads, doughs and porridges were stored at -18°C, and the dry products were stored at room temperature until they were analyzed. Before the analysis, all dry samples were mixed and homogenized in a blender (Braun 4250 Multimixture Machine, Braun Ab, Frankfurt/M, Germany). The breads were also homogenized and one sample of each was taken for analysis, for a total of five samples of one product. In the final P analysis by ICP–MS, the samples were analyzed in duplicate.

##### *Study II*

Legumes and cereals are important sources of plant-based protein and P, and thus, products from these food groups were included in the analyses (Table 8). Further, foodstuffs containing FAPs, i.e. baking powder-leavened bakery products as well phosphoric acid-containing cola beverages, were analyzed. Among cereals and cola beverages, several labels were analyzed to clarify P contents in the products of different manufacturers.

**Table 7.** *Samples, n's, sampling method, and possible freezing before analysis in Study I.*

Sample	n	Sampling method	Frozen (-18°C) before analysis
<i>Home-made/original products</i>			
Rye flour	5	Mixed	-
Rye dough	5	1/dough	+
Rye bread	5	1/bread, ground	+
Wheat flour	5	Mixed	-
Wheat dough	5	1/dough	+
Wheat bread	5	1/bread, ground	+
Soured wheat dough	5	1/dough	+
Soured wheat bread	5	1/bread, ground	+
Barley grit	5	Mixed, ground	-
Barley porridge	5	1/porridge, ground	+
<i>Bakery products</i>			
Wheat bread	5	1/bread, ground	+
Rye bread with potato	5	1/pack <sup>a</sup>	+
Rye bread	5	1/bread, ground	+

(+ frozen before analysis, - not frozen before analysis, <sup>a</sup> from every pack one bread was ground to one sample)

**Table 8.** *Products analyzed in Study II.*

Breads	Sweet bakery products	Seeds and beans	Drinks
Rye bread pool	Muffin pool <sup>a</sup>	Sesame seed (with hull)	Pepsi Max <sup>b</sup>
Rye crisp	Sweet bun pool	Tofu (firm)	Coca-Cola light <sup>b</sup>
Small rye bread containing potato 1	Cookie pool	Green bean	Coca-Cola <sup>b</sup>
Small rye bread containing potato 2		Green pea (frozen)	Cola pool <sup>b</sup>
Mixed grain bread with seeds		Chickpea (soaked)	Freeway Cola light <sup>b</sup>
		Red lentil	Beer
		Green lentil	

<sup>a</sup> Contains sodium polyphosphate as an additive

<sup>b</sup> Contains phosphoric acid as an additive

To improve representativeness of the analyses in Study II, the focus was on the nationally most popular products in each food category. Market shares of the

products were based on a substudy of the national dietary survey FINDIET 2007 (Reinivuo et al. 2010). A subsample (50%) of the participants (n =1025) of the FINDIET 2007 Study filled in a 5-day product purchase diary to investigate the purchase and use of voluntarily fortified foods. A total of 930 subjects (91%) returned the diary, 918 (90%) of which were of acceptable quality. Each food item was assigned with a food identifier from the Finnish food composition database Fineli<sup>®</sup>, enabling data classification. Market shares of each product were calculated by dividing the total amount (kg) of purchased product by the total amount of purchased food in the same food class.

Of the most popular trademarks, 2-7 of each were pooled for each sample item. The leading trademark in each sample category was analyzed as such. The chosen sample items were purchased from grocery stores in the Helsinki area. The number of purchased trademarks was twice the proportion of that item in the pool (40% in the pool, corresponding to 8 subsamples). The minimum was two subsamples for each pooled per trademark. The samples were homogenized in a blender, packed into polyethylene bags, and stored at -20°C until analysis. The samples were analyzed with five replicates.

#### **4.1.2. Study designs and hypotheses**

**Study I:** A new method for analysis of *in vitro* DP, indicating bioavailable P, was developed, and the *in vitro* DP and TP contents of certain differently processed cereals were determined. The hypothesis was that processed cereals contain more DP than the unprocessed ones due to the baking processes for rye and wheat breads and the effects of long processing time on barley porridges, all of which may degrade phytate resulting in higher DP contents.

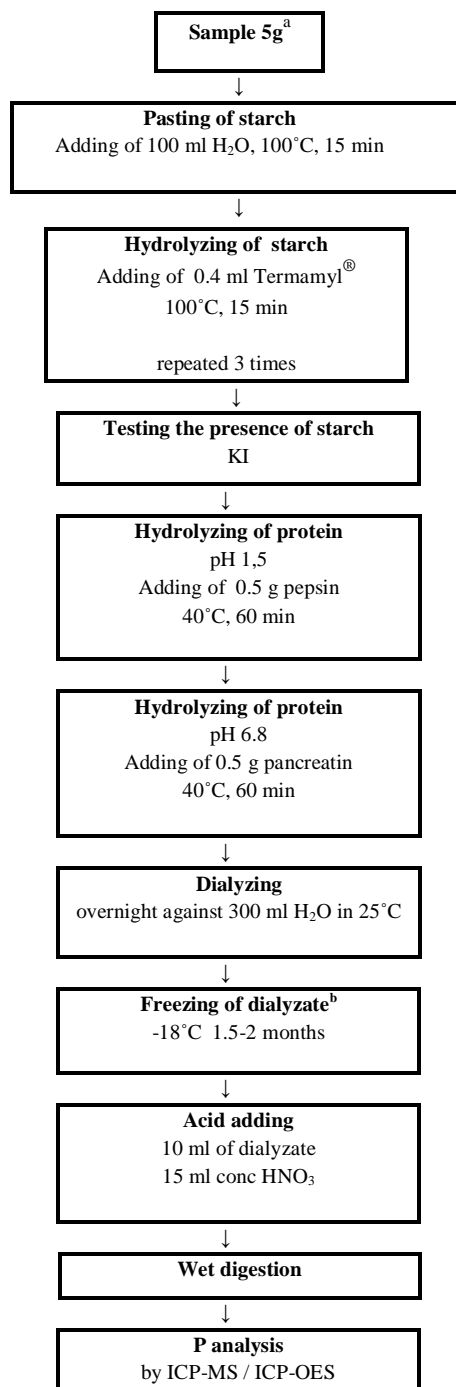
**Study II:** By the analysis method developed in Study I, the DP and TP contents of different plant-based products considered to be good dietary P sources were analyzed. The foodstuffs were chosen based on market shares, and the analyses included both natural and FAP-enhanced products. The hypotheses were that differences exist in DP and TP contents in the plant-based foodstuffs and the proportion of DP to TP is lower in plant-based FAP-free products than in products prepared with FAPs.



### 4.1.3. Methods

#### 4.1.3.1. Analysis of *in vitro* digestible phosphorus

The method for *in vitro* DP analysis is based on the method of Asp et al. (1983) for enzymatic assay of insoluble and soluble dietary fiber. There, the proteins and starch are degraded with alimentary enzymes and the mineral content in the remaining fiber is analyzed. Ekholm et al. (2000, 2003) developed the method for the analysis of soluble minerals. Figure 8 presents the flow-chart of the *in vitro* DP analysis. Here, a sample (5 g) was placed in a 100-ml flask and mixed with 50 ml of purified water (MilliQ, Millipore Corporation, Bedford, MA, USA), then cooked for 15 min at 100°C. Then 0.4 ml of heat-stable alpha-amylase Termamyl 120 L (Novozym A/S, Bagsvaerd, Denmark) was added to initiate starch degradation. Cooking for 15 min and adding of Termamyl (0.4 ml) was repeated two times. After the last addition, the mixture was cooked for a further 15 min. The presence of any unhydrolyzed starch was verified with I<sub>2</sub>/KI solution. If starch had been present, the color of the sample would have changed to violet. Instead, it turned yellow, indicating the presence of no starch. The amount of the enzyme was optimized to be as low as possible to avoid excess mineral contamination. The pH of the sample was adjusted to 1.5 by 1 M HCl. The pepsin (0.7 FIP-U/mg, E.C. 3.4.23.1, Merck, Darmstadt, Germany) dissolved in purified water (0.5 g in 10 ml) was added to the sample and the mixture was incubated at 40°C for 60 min on a continuous shaker. Before the pancreatin (8-U.S.P., Sigma–Aldrich, St Louis, MO, USA) was added, the pH was adjusted to 6.8 by 1 M NaOH. The pancreatin mixed in purified water (0.5 g in 10 ml) was added to the sample, which was incubated at 40°C for 60 min on a continuous shaker. After incubation, purified water was added to the sample until it weighed 100 g. Half of the sample (50 g) was transferred to a 60-cm-long dialysis tube (cut-off point 2.4 nm, size 5, Medicell International Ltd., London, UK). The samples were dialyzed in equilibrium dialysis against 300 ml purified water overnight with 10 drops of chloroform (Merck) to avoid bacterial growth. After dialysis the dialyzates were frozen (-18°C). The rest of the analysis differed between Studies I and II.



**Figure 8.** Flow-chart of enzyme preparations and digestible phosphorus analysis in *Studies I and II*. <sup>a</sup>some of the samples were ground before freezing bfreezing was used to make the process easier, HNO<sub>3</sub>=nitric acid, ICP-MS=inductively coupled plasma-mass spectrometry, ICP-OES=inductively coupled plasma-optical emission spectrometry, KI=potassium chloride.

## *Study I*

Two replicates of every dialyzate (10 ml) remained at room temperature with 15 ml of concentrated nitric acid ( $\text{HNO}_3$ ) (69.0–70.0%, J.T. Baker, Phillipsburg, NJ, USA) in a wet digestion tube and later processed in a wet digestion apparatus (Foss 2040 Digestor, Foss Tecator, Hillerod, Denmark). The wet digestion program for DP analysis is shown in Appendix (Table 2). Later, every sample was moved quantitatively to a 25-ml flask by flushing the wet digestion remnant with purified water and filling the flask to the 25-ml mark.

For the ICP–MS analysis, 400 ml of the sample were moved to an ICP tube. Rhodium (1 ml,  $c = 1000 \text{ mg/l}$ , AccuTrace™ Reference Standard Rhodium 1000 mg/l; AccuStandard, New Haven, CT, USA) was added as an internal standard, and the samples were diluted to 2%  $\text{HNO}_3$  (8.6 ml). Five P standard solutions (AccuTrace™ Reference Standard Phosphorus 1000 mg/ml; AccuStandard) with concentrations of 100, 200, 400, 1000 and 2000 mg/l were used. The standard curves were obtained at the above-mentioned concentrations. The samples were analyzed by using an ICP–MS device (Elan6000 PerkinElmerSciex, PerkinElmer, Waltham, MA, USA) using the isotope  $^{31}\text{P}$ . DP contents were calculated as mean per 100 g/product. Percentages of DP/TP (TP analysis described in Section 4.1.3.2.) were also calculated.

## *Study II*

Ten milliliters of the dialyzate was digested in a microwave oven with 10 ml of concentrated  $\text{HNO}_3$ . The sample was transferred to a 50-ml volumetric flask with purified water. The concentrations of P in the dialyzate were determined by ICP–OES (Thermo Scientific iCAP 6000 Series ICP Spectrometer, Waltham, MA, USA). The quantitation was performed by using an external calibration curve with five different concentrations. P was analyzed radially with wavelengths of 177.4, 178.2, 178.7, and 213.6 nm. The fat was removed from samples if the fat content was more than 6–8%. Petroleum ether (200 ml, bp: 60°C to 71°C) was added and the suspension was agitated with a magnetic stirrer at room temperature for 15 min. The solvent was filtered and the sample was air-dried at room temperature overnight, and analyzed by ICP–OES.

The results were calculated as means. DP contents of analyzed foods were calculated per 100 g of the product. The percentages of DP/TP (TP analysis described in Section 4.1.3.2.) were determined.

#### **4.1.3.2. Analysis of total phosphorus**

##### ***Study I***

In the TP analysis a homogenized sample (0.5 g) was weighed in a tube, and the sample remained in concentrated HNO<sub>3</sub> (10 ml) overnight before wet digestion. The wet digestion program is shown in Appendix (Table 2). After digestion the samples were treated as in DP analysis and the TP content was analyzed by an ICP-MS device using the isotope <sup>31</sup>P.

##### ***Study II***

The food sample (0.5 g) was digested in a microwave oven with 10 ml of concentrated HNO<sub>3</sub>. The sample was transferred to a 50-ml volumetric flask with purified water, and the content of P was analyzed with ICP-OES.

TP contents of analyzed foods in Studies I and II were calculated per 100 g of product.

#### **4.1.3.3. Assay quality control**

To avoid possible contamination, all glassware used in ICP analyses was acid-washed (HNO<sub>3</sub>/HCl) and rinsed with purified water (MilliQ). The enzymes used in DP analysis contain P, which could have affected the results. Therefore, the amounts of P in all enzymes (i.e. the background level) were analyzed from the same amount of enzymes as in the analysis without any substrate. These values were taken into account in the results. To test accuracy and precision of the analytical method, each determination set of the TP analysis included in

duplicate (in Study II: a single sample) two different reference samples, NIST 1567a wheat flour standard reference material (National Institute of Standards and Technology, Gaithersburg, MD, USA) and an in-house wheat flour standard (Saari and Paaso 1980), both containing a known amount of P. To estimate the repeatability of the method, in DP analysis an unofficial in-house white wheat flour sample was used in duplicate as a reference material in each determination set. All analyses were carried out at the Laboratory of General Chemistry, at the Department of Applied Chemistry and Microbiology.

The measurement uncertainty was calculated taking into account all possible sources of uncertainty in the analysis procedure and quantifying the uncertainty according to the statistical methods (Ellison et al. 2000). Approximate confidence interval of 95% was used, implying that 95% of the results are inside the limits of measurement uncertainty. The calculations were performed for both DP and TP.

#### **4.1.3.1. Statistical analyses**

The statistical analysis was performed by SPSS (Statistical Package for Social Sciences for Windows, version 13.0.1, SPSS Inc., Chicago, IL, USA). Statistical differences between the means of the TP and DP contents were tested by paired t-test (Study I). In Study I, the home-baked breads were also tested by paired t-test; the amount of DP in the bread and dough originating from 100 g of flour was compared with the DP amount in 100 g of flour. For barley, the difference between the amount of grits from which 100 g of porridge originated was compared with the amount of DP in 100 g of porridge. The differences were significant at  $p < 0.05$ . The differences between the means of the groups in the flours, doughs, and breads in Study I were tested by F-test, using one-way analysis of variance (ANOVA) for repeated measures. The normality of the data was first tested by Shapiro–Wilk’s test. The sphericity of the results of the groups was tested, and if the sphericity assumption had been violated, a Huynh–Feldt adjustment was used.

## **4.2. Human study (Study III)**

### **4.2.1. Subjects**

A randomly collected sample of 1920 subjects aged 37-47 years (50% males, 50% females) and living in the Helsinki area was derived from the Population Register Center in Finland. Subjects were contacted by mail in November 2009 (1<sup>st</sup> sampling) and February 2010 (2<sup>nd</sup> sampling) and invited to participate in the study. A total of 678 persons participated in the first visit to the research unit either in January or March 2010, and 653 of these attended the second visit during spring 2010 (discontinuation for unknown reasons, n=25). Subjects were middle-aged, and thus, possibly already had some changes in cIMT. The initial exclusion criterion was pregnancy, and also post-menopausal females and persons with impaired kidney functions (estimated GFR, i.e. eGFR, <60 ml/min) were excluded from the analyses due to their improper P metabolism.

The study protocol of the study was approved by the Helsinki Uusimaa Hospital District Ethics Committees. All subjects gave their informed consent to the procedures, which were in accord with the Helsinki Declaration. They also were informed that they could withdraw from the study and for any reason. After the study, subjects received information about their dietary intake relative to the nutritional recommendations as well as results on their vitamin D status (serum 25(OH)D concentrations) and tibial and radial bone mineral density compared with the age-matched population. Medical experts checked the results, and if health problems were perceived, the subjects were informed after the analyses and advised to contact a local doctor.

### **4.2.2. Study design and hypothesis**

The study design was cross-sectional and the study population comprised free-living, middle-aged males and premenopausal females, i.e. the general population. Associations among TP intake, P density of the diet, FAP intake, and cIMT were investigated. Subjects visited the research unit two times during

spring 2010. On the first visit, fasting blood samples were collected and subjects were advised to fill in the study form, which included food records, food frequency questionnaires (FFQ) concerning intakes of P, Ca, and vitamin D intake, and a background questionnaire. Before the second visit, subjects were advised to abstain from caffeine-containing products and smoking for four hours. During this visit the common cIMT was scanned by ultrasonography, blood pressure was measured by an automatic device, and subjects' height and weight were measured. Some other measurements (e.g. bone mineral density assessments) not included in the present study were also carried out; the power calculation of the study is based on bone mineral density assessments (see Section 4.2.3.4.). The hypothesis was that when taking into account the potential confounding factors, high P intake, especially in the forms of FAP, is associated with higher IMT, thus, high dietary P intake should be considered a CVD risk factor in the general population.

### **4.2.3. Methods**

#### **4.2.3.1. Dietary intake and background data collection**

Habitual dietary intake data of subjects were collected by 3-day food records, which included two weekdays and one weekend day. The subjects were instructed to maintain their normal food habits during the recording period and to record all foods, beverages, and dietary supplements immediately after consumption. Nutrient intake was calculated using a computer-based program (Diet 32 version 1.4.6.2, Aivo2000, Turku, Finland) based on the Finnish food composition database Fineli<sup>®</sup> (Institution of Health and Welfare, Helsinki, Finland). Approximately 400 new recipes were added to the program to ensure accurate calculation of nutrient intake. Vitamin D and Ca supplementation was taken into account in the calculations. P intake was calculated as total P intake (mg/d), and as P density of the diet [ $\text{P intake (mg/d)} / \text{energy intake (kJ/d)}$ ].

FAP intake calculations were based on one-month food use frequency data, which were collected by a validated FFQ concerning P intake, ranging from

“less than one portion per month” to daily portions (Kemi 2010). The FFQ included P sources in different food groups, distinguishing FAP-containing foodstuffs from natural P-containing foodstuffs, and also including P sources not consumed every day. For FAP intake calculations, products known to contain FAP were chosen and separated these into food groups (meat products like marinated meat, sausages, and cold cuts; cola beverages; processed cheeses, Table 9). FAP intake from different sources was calculated from the portions of FAP-containing foodstuffs as grams and recorded on the FFQ, using the maximum amount of FAP allowed in the foodstuff according to European Union regulations (meat products 5 g/kg; cola beverages 700 mg/L; processed cheeses 20 g/kg (European Union 2011)). Only these products in which the amount of added FAP is regulated were included in the analysis (for example, starch-based FAPs were not included). Cookies were excluded from the calculations because some do not contain FAPs. The subjects were divided into tertiles of intake in each FAP group (meat products, cola beverages, processed cheeses), with score 0 indicating the lowest intake tertile, score 1 the middle tertile, and score 2 the highest tertile, and the scores from different FAP sources were summed for each subject; thus, each person was given a score from zero to six. Since no one received a score of 0, thus, the scoring was 1-6 and divided the subjects into six groups of unequal size. However, the final group sizes were sufficient for analysis of covariance (ANCOVA).

Background data were collected by a questionnaire covering disease history and lifestyle factors such as smoking status (current/former or non-smoker), physical activity, and state of menopause. Body mass index (BMI) was calculated as weight (kg) / height (m)<sup>2</sup>. All self-reported forms were checked by the researchers, and additional information was requested if needed.



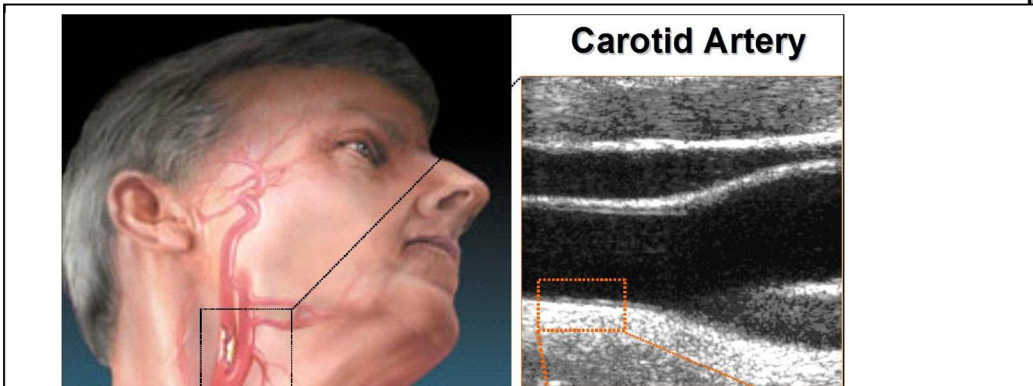
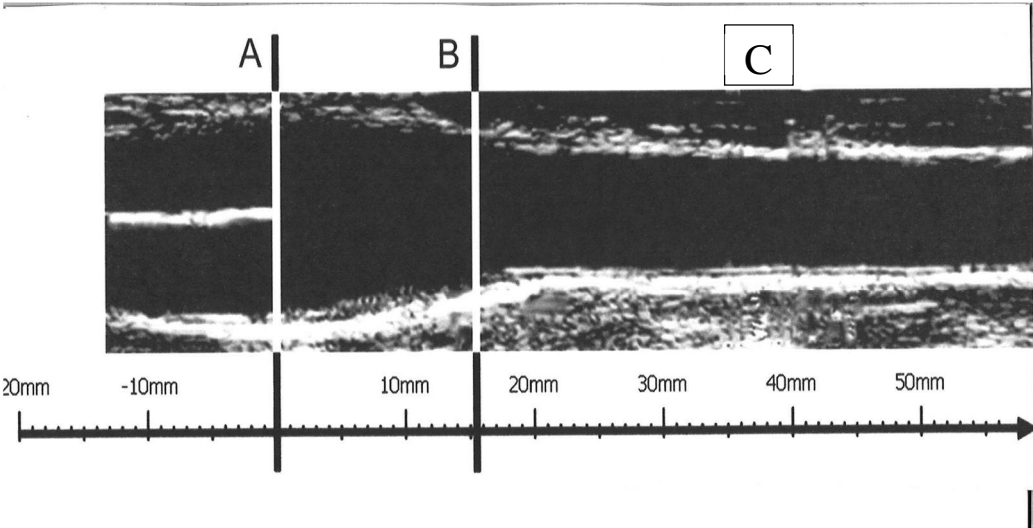
**Table 9.** Food additive phosphate (FAP) -containing products included in FAP intake calculations.

FAP-containing product	Portion size
<i>Processed meat products</i>	
Processed, marinated chicken, turkey, beef, pork, or other meat	1 steak (130 g)
Liver pâté and liver sausage, regular and light	1 tablespoon (15 g)
Sausages / frankfurters, regular and light	10 cm slice of big sausage, 3 frankfurters or 1 barbeque sausage (100 g)
Meat cold cuts, regular and light	1 slice (15 g)
Sausage-style cold cuts, regular and light	1 slice (15 g)
<i>Processed cheeses</i>	
With different fat contents: >24%, 20-24%, 9-19%, and 9%	1 tablespoon or one individually packed slice (15 g)
<i>Cola beverages</i>	
Regular and light	One glass (200 ml)

#### **4.2.3.2. Measurement of the carotid intima-media thickness and blood pressure**

Common carotid artery IMT was measured by a semi-automatic measurement program using high-resolution ultrasonography (Esaote MyLab30Gold, Firenze, Italy). The image is based on high ultrasound frequencies, which reflect from the tissues in different ways. In practice, this means that the thicker the white line, i.e. the intima-media in the image, the more calcification occurs (Figure 7). However, this technique does not distinguish between the intimal and medial layers of the artery (O’Leary and Bots 2010). Ultrasound provides a two-dimensional image, thus, either the far or near wall can be measured. The consensus statement proposed that cIMT be used to measure the far wall of the common carotid artery. Measurement of the near wall is not as reliable because the ultrasound reflects from the adventitia to the intima, i.e. from the more echoing part to the less echoing part, and the wall thickness can be suboptimal (Stein et al. 2008). The thickness of the cIMT varies depending on cardiac cycle, being thickest at the end of diastole and thinnest at the peak of the systole; thus,

measurement should be done in all subjects at the same time phase (O’Leary and Bots 2008, Holewijn et al. 2010).



**Figure 7.** *Above: Ultrasound image of carotid artery. Intima-media thickness (IMT) is commonly measured 10 mm left of bifurcation. Left from A = internal carotid artery, B = bifurcation (bulb), C = common carotid artery (adapted from Lorenz et al. (2007)).*

Here, IMT was measured from a 1-cm sample from the bifurcation (bulb) with an 8- or 10-mHz linear array transducer by three experienced sonographers. The software measured the far wall thickness bilaterally for the 1-cm segment. The measurement was carried out on the left and right carotid in duplicate with the

subject in a supine position (Stein et al. 2008). The mean intima-media complex thickness was automatically calculated in micrometers in diastolic phase scans. The averages of these four measurements were used in the analyses. Repeatability of the measurements was ensured by rescanning 18 participants within one week of the first IMT scanning. In addition, cIMT of 15 participants was measured by two sonographers. The coefficients of variation between the measurements obtained by the same sonographer (intra CV%) and between measurements obtained by different sonographers (inter CV%) are shown in Table 10. Systolic and diastolic blood pressures were measured after the IMT measurement in supine position in duplicate by an automatic device (Omron MIT Elite Digital Automatic Blood Pressure Monitor, OMRON Healthcare Europe B.V., Hoofddorp, the Netherlands), and the second result was used in the analyses. Pulse pressure was calculated as systolic – diastolic.

#### **4.2.3.3. Laboratory analyses**

Twelve-hour fasting blood samples were collected between 7:30 am and 9:15 am during the first visit. For serum samples, Venoject gel tubes and for plasma/whole blood EDTA tubes were used. Serum/plasma was extracted from blood (except whole-blood samples) by centrifugation at 3000 rpm for 15 min within half an hour after sampling and stored immediately after sampling at -20°C to -70°C until analysis. The samples were collected in the morning due to the circadian rhythm of Pi and its metabolites (Calvo et al. 1991) as well as during wintertime to avoid the effects of sunlight on serum 25(OH)D concentrations (Lamberg-Allardt et al. 1983).

Table 10 describes the assessments carried out, the devices and methods used, and the inter and intra CV%'s. LDL-C/high-density lipoprotein cholesterol (HDL-C) ratio was calculated as LDL-C/HDL-C. eGFR was determined by using the Cockcroft-Gault formula (Poggio et al. 2005). All analyses were conducted at the Laboratory of Nutrition, at the Department of Food and Environmental Sciences.

**Table 10.** *Inter and intra coefficients of variation percentages (inter CV%; intra CV%) of the laboratory analyses and IMT measurements.*

Assessment	Device	Method	inter CV%	intra CV%
S-Total cholesterol	Konelab20 <sup>1</sup>	photometric	4.6	4.6
S-HDL-C	Konelab20 <sup>1</sup>	photometric	4.6	4.6
S-LDL-C	Konelab20 <sup>1</sup>	photometric	4.6	4.6
S-Triglycerides	Konelab20 <sup>1</sup>	photometric	4.6	4.6
S-Creatinine	Konelab20 <sup>1</sup>	photometric	4.6	4.6
S-Glucose	Konelab20 <sup>1</sup>	photometric	4.6	4.6
S-hs-CRP	Konelab20 <sup>1</sup>	photometric	4.6	4.6
S-Calcium	Konelab20 <sup>1</sup>	photometric	4.6	4.6
S-Phosphate	Konelab20 <sup>1</sup>	photometric	4.6	4.6
HbA1c%	Konelab20 <sup>1</sup>	photometric	4.6	4.6
S-25(OH)D*	IDS Kit <sup>2</sup>	enzyme immuno assay	2.7	3.2
S-iPTH	Immulate1000 <sup>3</sup>	immunoluminescence	8	<5.5
S-Insulin	Immulate1000 <sup>3</sup>	immunoluminescence	<4.9	<6.2
P-FGF-23	Immutopics kit <sup>4</sup>	enzyme-linked immuno assay	<5.5	<7.7
IMT	MyLab30Gold <sup>5</sup>	ultrasound	9.2	6.6

<sup>1</sup> Thermo Clinical Labsystems, Espoo, Finland

<sup>2</sup> Immunodiagnosics Systems Ltd., Boldon, UK

<sup>3</sup> Siemens Healthcare Diagnostics, Malvern, PA, USA

<sup>4</sup> Immutopics INC., San Clemente, CA, USA

<sup>5</sup> Esaote MyLab30Gold, Firenze, Italy

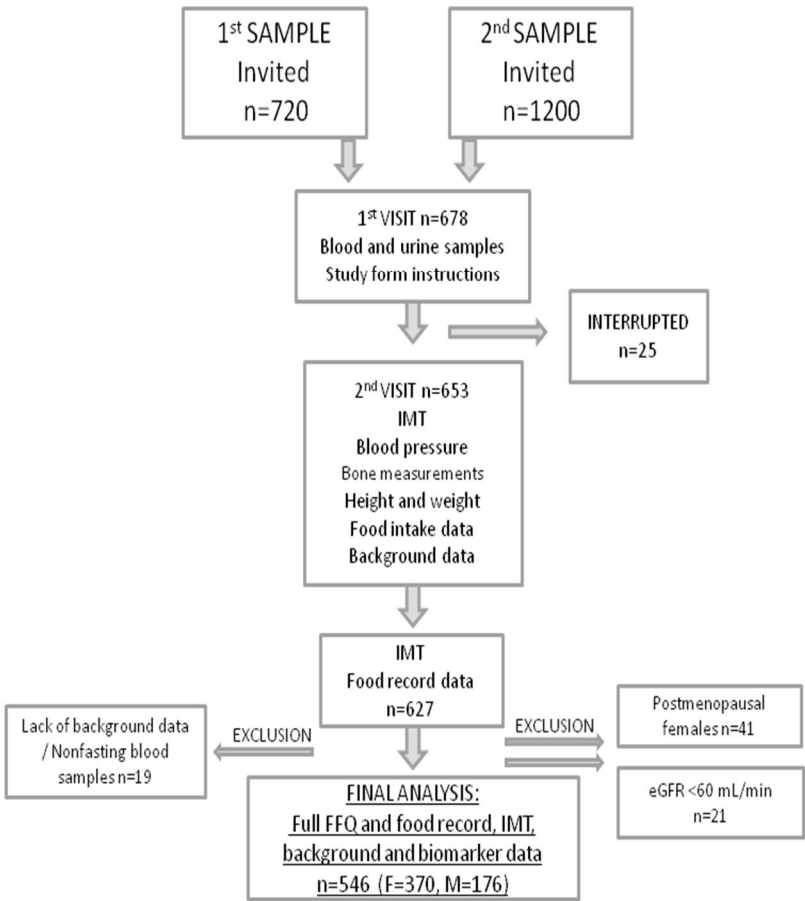
\*reproducibility ensured by Vitamin D External Quality Assessment Scheme (DEQAS)

HDL-C high density lipoprotein cholesterol; LDL-C low density lipoprotein cholesterol; hs-CRP high sensitive C-reactive protein; HbA1c% glycosylated haemoglobin; 25(OH)D 25-hydroxy vitamin D; iPTH intact parathyroid hormone; FGF23 fibroblast growth factor 23; IMT intima-media thickness

#### 4.2.3.4. Final sample

IMT and food intake data were successfully collected from 627 persons. Of these, postmenopausal females (n=41, data collected by a questionnaire) were excluded from the analyses, as were persons with moderate renal dysfunction (estimated glomerular filtration rate, eGFR<60 ml/min) (n=21). In addition, 19 persons were excluded because they did not complete the study due to lack of

background data or non-fasting blood samples. The final analyses included 370 females and 176 males for whom full nutrition, IMT, background, and biomarker data were available. Sampling and study protocol are shown in Figure 9.



**Figure 9.** Flow-chart of sampling and protocol of Study III. IMT= intima-media thickness, eGFR=estimated glomerular filtration rate, FFQ=food frequency questionnaire, F=female, M=male.

#### 4.2.3.5. Statistical analyses

The power of the study (80%,  $\alpha=0.05$ ) was calculated basing on the primary variable of the main study, to find a 4% difference in forearm bone mineral density between the highest and lowest P intake tertiles. 800 subjects (400 females, 400 males) were expected to participate. Thus, taking into account the estimated initial drop-out rate of 40%, 720 subjects were invited to the first sampling. However, the drop-out rate was higher than expected (about 60%) and to the second sampling 1200 subjects were invited to ensure sufficient power of the study including the drop out rate during the study. For IMT analyses no power calculations were performed due to lack of reference values.

Statistical analysis was performed using PASW Statistics version 18.0.2 (IBM, Armonk, NY, USA). The normality and homogeneity of the data were verified. Equality of the variances in quintiles and FAP groups was assessed by Levene's test.

Correlations among IMT, TP, P density of the diet, and FAP intake and potential confounding factors were assessed by Pearson correlation coefficients (data not shown). The covariates were chosen depending on their correlation with IMT and P intake and their contextual significance. Highly intercorrelated ( $r>0.2$ ) confounding factors were not used in the same models. Due to the high correlation between Ca and P intake, Ca was excluded from the covariates. BMI correlated with IMT, TP intake, and almost all potential covariates, and thus, BMI was also excluded from the covariates. Serum LDL-C/HDL-C ratio was used because it predicts IMT progression better than HDL-C or LDL-C alone (Enomoto et al. 2011). IMT scanning results of one sonographer differed significantly from the others; thus, sonographer class was coded as a dummy variable (0 for non-differing sonographer, 1 for differing sonographer) and used as a random factor in ANCOVA. Smoking status was also coded as dummy (0 for never-smoker, 1 for former or current smoker). The final covariates were sex (when analyzing all subjects), age, smoking status, LDL-C/HDL-C ratio, and IMT sonographer as a random factor. Pulse pressure was also included in some models, but this did not strengthen the results (data not shown). P value less than 0.05 was considered statistically significant.

Differences between quintiles of TP intake/P density of the diet and six FAP groups, considering potential confounding factors, were assessed by analysis of variance (ANOVA, unadjusted for covariates). ANCOVA was used to analyze differences in IMT between quintiles of TP/P density of the diet and FAP groups. Especially under interest was to determine whether high TP/P density of the diet/ FAP intake is associated with higher IMT, or are there differences in IMT between high and low P intake. Tests for significant linear trends in associations between P variable intakes and IMT were performed by contrast analysis.

## 5. Results

### 5.1. Study I

#### 5.1.1. Usefulness of the method for analysis of *in vitro* digestible phosphorus

In the DP analysis, the mean P content of the unofficial in-house white wheat flour reference samples (n=32) was 49.7 mg P/100 g (SD 9.0 mg). Due to the newness of the analysis method, no earlier results of DP content in the reference material were available and these results only estimate the repeatability of the method. The accuracy and precision of the TP analysis was tested by a certified reference sample (NIST 1567a, wheat flour, n=10) and by an unofficial inhouse wheat flour reference sample (n=10). The mean values ( $\pm$ SD) for the references were 133.4 $\pm$ 8.6 mg P/100 g and 342.7 $\pm$ 8.6 mg P/100 g, respectively. The NIST 1567a value was in accordance with the certified range (134 $\pm$ 6 mg P/100 g) analyzed by a standard methodology (National Institute of Standards and Technology) by the manufacturer and acceptable (361 $\pm$ 8 mg P/100 g) for the in-house reference (Saari and Paaso 1980). For the P analysis by ICP-MS, the limit of detection was 0.3 mg/l and the limit of quantitation 3 mg/l (equivalent to 60 mg/kg in the original sample). The values were based on 14 standard blanks and were calculated as 3\*SD and 10\*SD of the standard blank, respectively. The quantitation was made by using external calibration with 5 points between 0 and 2000 mg/l. The curve type linear through zero was used. The typical correlation factor of the standard curve was 0.99999 and the linear measurement range was from 0 mg/l to 6000 mg/l. The calculated uncertainty for the *in vitro* DP analysis was 7.4%, and for the TP analysis 6.4%.

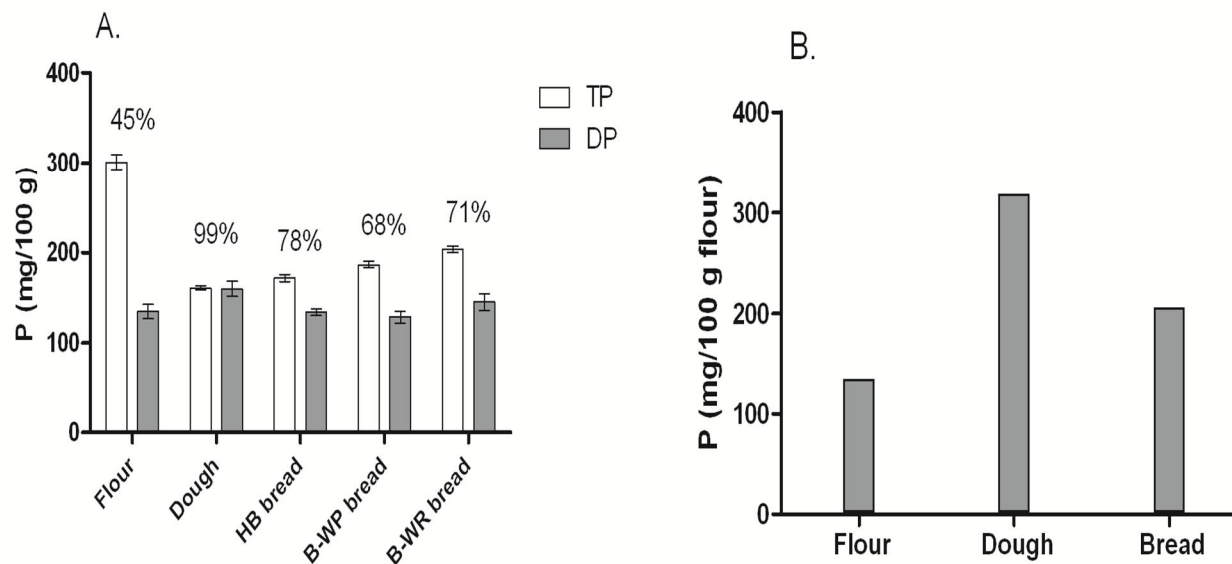


### **5.1.2. *In vitro* digestible and total phosphorus contents in differently processed cereals**

The absolute values of DP and TP contents in analyzed foodstuffs within measurement uncertainty are shown in Appendix, Table 3. In rye samples, TP content was higher in rye flour than in rye breads (Figure 10A). In rye breads the percentage DP relative to TP was similar (68–78%) in all breads analyzed. Differences between the DP contents of home-baked and bakery breads were not significant ( $p>0.05$ ). The amount of DP in the rye sourdough was almost 100%, indicating that some of DP has become insoluble in the baking process, when comparing to the DP contents which were lower in bread. The high DP amount in dough can also be explained by the measurement uncertainty. Figure 10B shows DP contents in rye dough and bread originating from 100 g of flour. When the results of DP in dough and bread originating from 100 g of flour were tested by paired t-test, the differences were significant ( $p<0.001$ ) between all pairs compared (flour-dough, flour-bread, dough-bread).

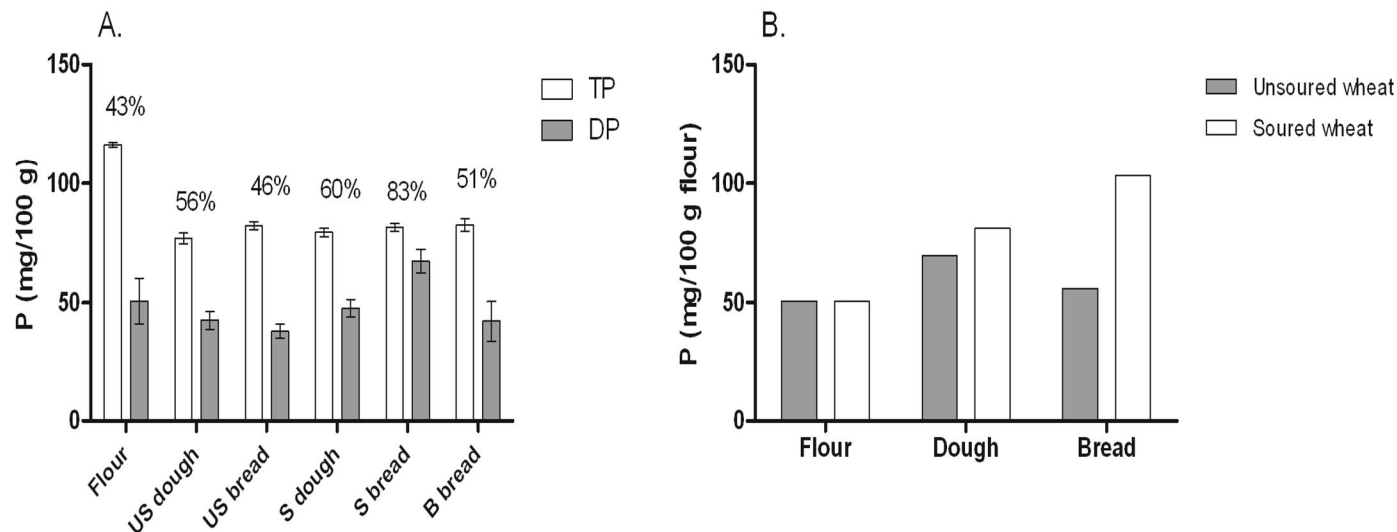
In wheat samples, DP content was 46–51% of TP in wheat breads, 56–83% in doughs, and 43% in flour (Figure 11A). The analyzed non-soured wheat cereals contained lower percentages of DP than soured cereals. Figure 11B shows DP contents in wheat dough and bread originating from 100 g of flour. When DP contents in dough and bread originating from 100 g of flour were compared by paired t-test, the differences were significant ( $p<0.001$ ) between all pairs compared (flour–dough, flour–bread, dough–bread), except for wheat flour – non-soured bread ( $p=0.158$ ). In wheat flour – non-soured dough, the differences were also significant ( $p=0.002$ ). However, DP, as well TP contents in wheat products were lower than in rye samples.

In processing of barley, the 3-h baking time degraded the phytate in large amounts and increased the DP content from 29% in grits to 69% in porridge ( $p<0.001$ ) (Figure 12A). Figure 12B shows DP contents in barley products considering the grits used for 100 g of porridge; there was an almost 2.5-fold increase in the DP contents. Like in wheat samples, the DP and TP contents in barley samples were lower than in rye samples.



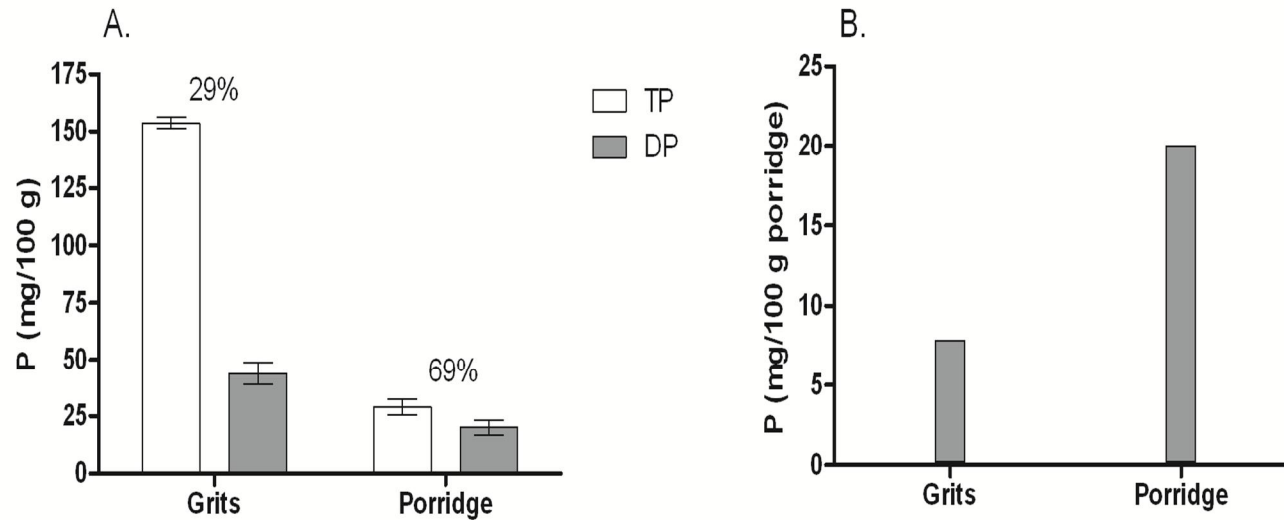
**Figure 10. Panel A:** Total and digestible phosphorus contents in analyzed **rye products**. Error bars show standard deviation. Percentages indicate digestible phosphorus/total phosphorus. TP total phosphorus = DP digestible phosphorus; HB = home-baked bread; B-WP bread = bakery bread also containing wheat and potato; B-WR bread = bakery whole rye bread.

**Panel B:** Digestible phosphorus contents in **rye products** considering the amount of dough and bread originating from 100 g of flour.



**Figure 11. Panel A:** Total and digestible phosphorus contents in analyzed *wheat products*. Error bars show standard deviation. Percentages indicate digestible phosphorus/total phosphorus. TP = total phosphorus; DP = digestible phosphorus; US dough = unsoured dough; US bread = unsoured bread; S dough = soured dough; S bread = soured bread; B bread = bakery bread.

**Panel B:** Digestible phosphorus contents in *wheat products* considering the amount of dough and bread originating from 100 g of flour.



**Figure 12. Panel A:** Total and digestible phosphorus contents in analyzed *barley products*. Error bars show standard deviation. Percentages indicate digestible phosphorus/total phosphorus. TP = total phosphorus; DP = digestible phosphorus.

**Panel B:** Digestible phosphorus contents in *barley products* considering the grits used for 100 g porridge.

## 5.2. Study II

### 5.2.1. *In vitro* digestible and total phosphorus contents in the analyzed plant-based foodstuffs

Table 11 presents the TP and DP contents of analyzed plant foods (Section A: Bakery products, Section B: Legumes and seeds, Section C: Beverages). Percentages of DP/TP contents are shown in Figure 13.

The highest P contents were found in rye crisp (TP 291 mg/100 g; DP 191 mg/100 g) (Table 11A). The TP contents of all other breads, except in rye crisp, were similar (189-208 mg/100 g), but DP contents of the breads varied more (54-123 mg/100 g). Some differences were found in DP contents between two very similar small rye breads containing potato (89 vs. 54 mg/100 g). FAP-containing muffins had relatively high contents of both TP (212 mg/100 g) and DP (201 mg/100 g), as well the percentages of DP (Figure 13). Cookies and sweet buns had lower TP and DP contents than muffins (116-125 mg/100 g TP, 43-60 mg/100 g DP, respectively).

TP contents among the analyzed legume and seed products varied between 57 (green beans) and 667 mg/100 g (sesame seeds) (Table 11B). The lowest DP content was found in green bean (24 mg/100 g), which had, however, the highest percentage of DP (42%). Apparently, the lowest percentage of DP/TP was present in sesame seeds (6%) (Figure 13).

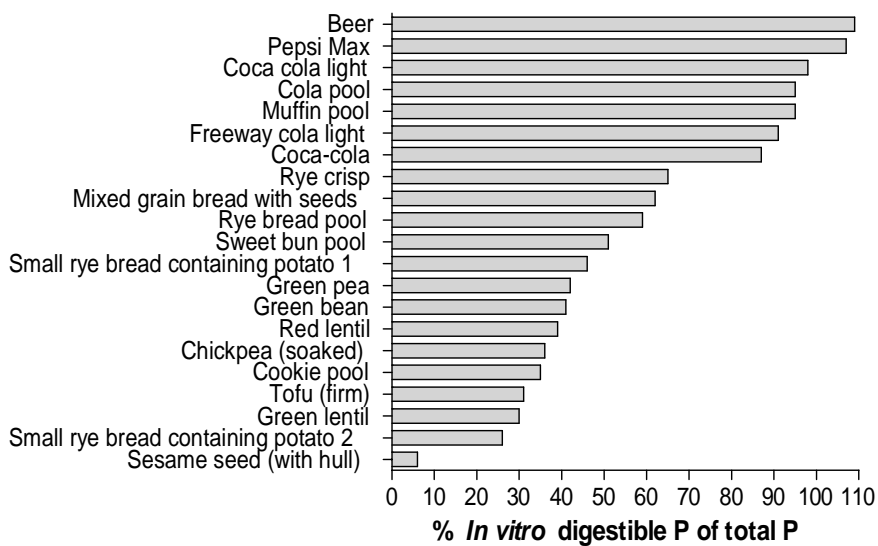
DP and TP contents in all cola beverages and beer were of lowest of the foods analyzed in this study (Table 11C). Almost all of the P in these beverages was *in vitro* digestible P (beer 22 mg/100 g, colas 10-16 mg/100 g). The percentages of DP for some beverages exceeded 100%, probably due to the inaccuracy of the analysis method with regard to low P contents (Table 11C). FAP-containing cola drinks had high percentages of DP.

**Table 11.** *Total phosphorus (TP) and in vitro digestible phosphorus (DP) contents of the analyzed plant foods.*

<b>Product</b>	<b>TP (mg/100 g)</b>	<b>DP (mg/100 g)</b>
<b><i>A: Bakery products</i></b>		
Rye bread pool	208	123
Rye crisp	291	191
Small rye bread containing potato 1	192	89
Small rye bread containing potato 2	206	54
Mixed grain bread with seeds	189	116
Muffin pool <sup>a</sup>	212	201
Sweet bun pool	116	60
Cookie pool	125	43
<b><i>B: Legumes and seeds</i></b>		
Sesame seed (with hull)	667	42
Tofu (firm)	164	51
Green bean	57	24
Green pea (frozen)	118	50
Chickpea (soaked)	149	53
Red lentil	432	167
Green lentil	400	120
<b><i>C: Beverages</i></b>		
Pepsi Max <sup>b</sup>	14	15
Coca-Cola light <sup>b</sup>	13	13
Coca-Cola <sup>b</sup>	19	16
Cola pool <sup>b</sup>	17	16
Freeway Cola light <sup>b</sup>	11	10
Beer	21	22

<sup>a</sup> Contains sodium polyphosphate as an additive

<sup>b</sup> Contains phosphoric acid as an additive



**Figure 13.** Percentages of *in vitro* digestible phosphorus (P) to total P in analyzed plant-based foods.

## 5.3. Study III

### 5.3.1. Characteristics of subjects

The study population was 37-to-47-year old, slightly overweight, and normotensive. Two-third were females, half of the population was current or earlier smokers. The subjects were to some extent hypercholesterolemic, otherwise healthy considering the assessed biomarkers (Tables 12 and 13). The nutrient intakes were according to the recommendations; however, the mean P intake exceeded the recommendations (Table 12).

**Table 12.** Background and nutrient intake data of study subjects. Values are presented as mean  $\pm$  SD.

Variable	All (n=546)	Females (n=370)	Males (n=176)
<i>Background data</i>			
Intima-media thickness ( $\mu\text{m}$ )	552 $\pm$ 69	544 $\pm$ 70	567 $\pm$ 66
Body mass index ( $\text{kg/m}^2$ )	26.8 $\pm$ 4.9	26.6 $\pm$ 5.2	27.2 $\pm$ 3.9
Age (years)	41.9 $\pm$ 2.8	41.9 $\pm$ 2.7	42.1 $\pm$ 3
Sex (% females)	67 $\pm$ 47	100	-
Current or former smoker (%)	48 $\pm$ 50	45 $\pm$ 50	55 $\pm$ 50
Systolic blood pressure (mmHg)	125 $\pm$ 14	122 $\pm$ 14	129 $\pm$ 13
Diastolic blood pressure (mmHg)	77 $\pm$ 11	76 $\pm$ 11	80 $\pm$ 11
Pulse pressure (mmHg)	48 $\pm$ 6	47 $\pm$ 6	50 $\pm$ 6
All physical activity (mins/week)	479 $\pm$ 380	515 $\pm$ 397	406 $\pm$ 332
<i>Nutrient intake data</i>			
Energy intake (kJ/d)	8413 $\pm$ 1994	7944 $\pm$ 1807	9398 $\pm$ 2014
Fat intake (g/d)	80 $\pm$ 26	76 $\pm$ 26	88 $\pm$ 25
Saturated fat intake (g/d)	29 $\pm$ 11	28 $\pm$ 11	32 $\pm$ 11
Phosphorus intake (mg/d)	1617 $\pm$ 428	1532 $\pm$ 378	1795 $\pm$ 469
Calcium intake (mg/d) *	1199 $\pm$ 453	1195 $\pm$ 435	1207 $\pm$ 489
Vitamin D intake ( $\mu\text{g/d}$ ) *	9.1 $\pm$ 8.6	9.4 $\pm$ 9.3	8.4 $\pm$ 6.8
Phosphorus density of the diet (mg/MJ/d)	194 $\pm$ 36	195 $\pm$ 37	192 $\pm$ 34
FAP intake score	3.2 $\pm$ 1.4	3.1 $\pm$ 1.3	3.6 $\pm$ 1.4

\* supplements included; FAP=food additive phosphate



**Table 13.** Biomarker data of study subjects. Values are presented as mean  $\pm$  SD.

Variable	All (n=546)	Females (n=370)	Males (n=176)
S-Pi (mmol/l)	1.09 $\pm$ 0.15	1.1 $\pm$ 0.15	1.06 $\pm$ 0.16
S-Ca (mmol/l) <sup>a</sup>	2.09 $\pm$ 0.07	2.09 $\pm$ 0.07	2.1 $\pm$ 0.07
P-FGF-23, C-terminal (RU/ml)	78.5 $\pm$ 84.7	85.1 $\pm$ 101.7	64.9 $\pm$ 20.1
S-PTH (pg/ml)	55 $\pm$ 25.2	56.9 $\pm$ 26	51 $\pm$ 23
S-25(OH)D (nmol/l)	55.4 $\pm$ 19.5	56.4 $\pm$ 20	53.4 $\pm$ 18.3
S-Total cholesterol (mmol/l)	5.45 $\pm$ 0.9	5.29 $\pm$ 0.84	5.77 $\pm$ 0.93
S-HDL-C (mmol/l)	1.51 $\pm$ 0.4	1.61 $\pm$ 0.39	1.33 $\pm$ 0.37
S-LDL-C (mmol/l)	2.94 $\pm$ 0.79	2.75 $\pm$ 0.72	3.34 $\pm$ 0.79
LDL-HDL ratio	2.13 $\pm$ 0.94	1.85 $\pm$ 0.77	2.71 $\pm$ 0.98
S-Triglycerides (mmol/l)	1.3 $\pm$ 0.73	1.14 $\pm$ 0.61	1.62 $\pm$ 0.86
eGFR (ml/min)	91.5 $\pm$ 20.9	85.6 $\pm$ 19.5	103.5 $\pm$ 18.6
S-HS-CRP (mg/l)	2.07 $\pm$ 4.47	2.13 $\pm$ 4.22	1.93 $\pm$ 4.97
S-Glucose (mmol/l)	5.08 $\pm$ 0.6	5 $\pm$ 0.48	5.24 $\pm$ 0.78
HbA1c %	5.06 $\pm$ 0.26	5.04 $\pm$ 0.24	5.11 $\pm$ 0.28

S=serum; P=plasma; Pi = phosphate; Ca = calcium; FGF-23 = fibroblast growth factor 23; PTH = parathyroid hormone;

PTH = parathyroid hormone; 25(OH)D = 25-hydroxy vitamin D; HDL-C = high-density lipoprotein cholesterol;

LDL-C = low-density lipoprotein cholesterol; eGFR = estimated glomerular filtration rate; HS-CRP = high-sensitivity

C-reactive protein; HbA1c % = glycosylated hemoglobin (% of total hemoglobin) <sup>a</sup> adjusted for albumin

The correlations between IMT, P intake, P density of the diet, and FAP intake, and potential confounding factors were assessed to define the covariates for the ANCOVA analysis (data not shown). Further, subjects were divided into quintiles (FAP intake; six groups) to find out the differences between those groups concerning the regulators of P metabolism; particular attention was paid to S-Pi, P-FGF23, S-iPTH, and S-25(OH)D concentrations as well as to Ca:P intake ratios (ANOVA, unadjusted for covariates). These analyses were only performed for all subjects. Quintile/group sizes and P intakes, P densities of the diet, and FAP intakes scores are described in Table 14.

**Table 14.** *Quintile/group sizes and ranges of P intakes, P densities of diet, and FAP intakes scores in quintiles/groups.*

	1st	2nd	3rd	4th	5th	6th
All subjects (n)	109	109	109	109	110	-
P intake (mg/d)	0-1272	1273-1462	1463-1665	1666-1962	1963-3205	-
Females (n)	74	74	75	73	74	-
P intake (mg/d)	0-1232.7	1233-1390	1391-1563	1564-1795	1796-2800	-
Males (n)	34	35	35	36	35	-
P intake (mg/d)	0-1443	1444-1610	1611-1850	1851-2163.9	2164.0-3205	-
All subjects (n)	109	109	109	109	110	-
P density of diet (mg/MJ/d)	0-162.1	162.2-182.8	182.9-200.85	200.86-222.1	222.2-320	-
Females (n)	74	74	75	73	74	-
P density of diet (mg/MJ/d)	0-164.5	164.6-183.0	183.1-203.0	203.1-224.0	224.1-310	-
Males (n)	35	35	35	36	35	-
P density of diet (mg/MJ/d)	0-160	161-182	183-198	199-218.0	218.1-320	-
All subjects (n)	66	111	151	109	85	29
FAP score	1	2	3	4	5	6
Females (n)	45	77	101	73	55	20
FAP score	1	2	3	4	5	6
Males (n)	16	39	54	28	33	10
FAP score	1	2	3	4	5	6

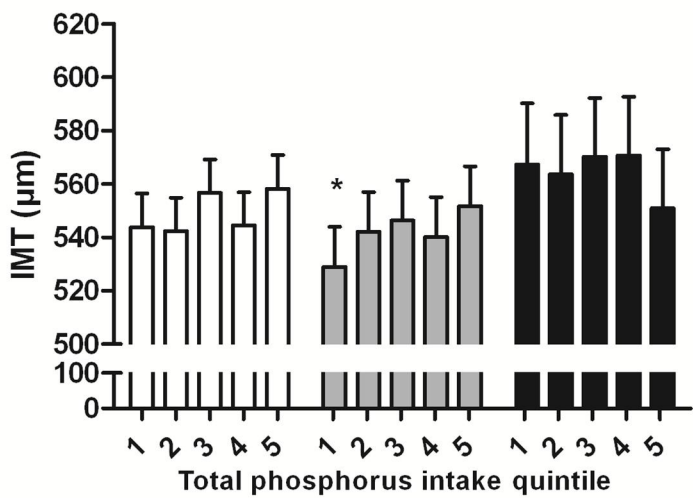
No significant differences in S-Pi ( $p=0.739$ ), P-FGF-23 ( $p=0.511$ ), S-iPTH ( $p=0.173$ ), or S-25(OH)D ( $p=0.073$ ) concentrations emerged between TP quintiles. Ca:P ratios between quintiles differed almost significantly with a tendency towards higher Ca:P ratio with higher TP intake ( $p=0.059$ ). S-Pi and P-FGF-23 concentrations did not differ significantly across P density quintiles ( $p=0.731$  and  $p=0.187$ , respectively). S-iPTH concentrations were lower ( $p=0.045$ ) and S-25(OH)D concentrations higher ( $p=0.004$ ) with higher P density. Ca:P ratios were lower with lower P density ( $p=0.018$ ). Between FAP groups, no significant differences emerged in P-FGF-23 ( $p=0.499$ ), S-iPTH ( $p=0.313$ ), S-25(OH)D concentrations ( $p=0.418$ ), or Ca:P ratios

( $p=0.266$ ), but S-Pi concentrations were negatively associated with FAP intake ( $p=0.006$ ).

**5.3.2. Associations between phosphorus intake variables and carotid intima-media thickness**

***Total phosphorus intake***

No significant association was found between TP intake and IMT (for all  $p=0.233$ , for females  $p=0.293$ , ANCOVA adjusted for sex [when analyzing all subjects], age, LDL-C/HDL-C ratio, smoking status, and IMT sonographer class) (Figure 14). Neither was there any linear trend in associations between TP intake and IMT among all subjects ( $p=0.136$ , contrast analysis), but among females an almost significant positive linear trend was present ( $p=0.072$ , contrast analysis), and the 1<sup>st</sup> and 5<sup>th</sup> intake quintiles differed significantly ( $p=0.035$ ). No significant differences were found among males ( $p>0.05$ ).

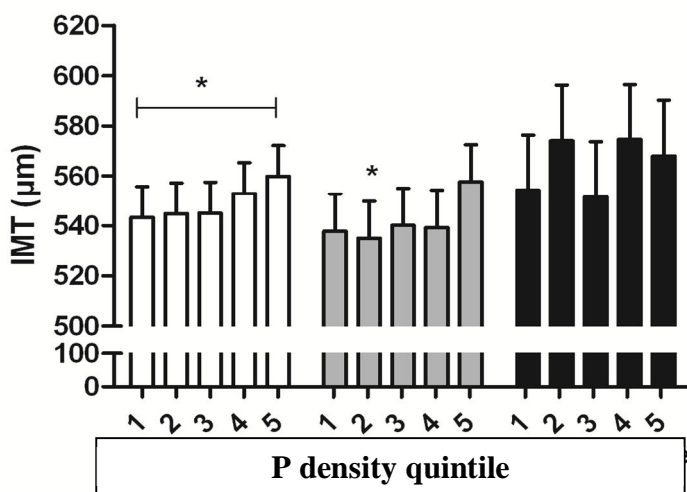


**Figure 14.** Mean carotid intima-media thickness (IMT) with upper limit of 95% confidence interval in TOTAL PHOSPHORUS (TP) intake quintiles for ALL SUBJECTS (WHITE SCALE,  $p$  for all 0.233,

ANCOVA), for **FEMALES** (**GRAY SCALE**,  $p$  for all 0.293, ANCOVA) and for **MALES** (**BLACK SCALE**,  $p$  for all 0.719, ANCOVA). Statistically significant differences from the highest TP intake quintile (ANCOVA) are shown with an asterisk (\*  $p < 0.05$ ) (between all other groups  $p > 0.05$ ).

### ***Phosphorus density of the diet***

No significant association existed between P density of the diet and IMT (for all  $p = 0.289$ , for females  $p = 0.231$ , ANCOVA adjusted for sex [when analyzing all subjects], age, LDL-C/HDL-C ratio, smoking status, and IMT sonographer class) (Figure 15). However, in between-quintile comparison among all subjects the 1<sup>st</sup> and 5<sup>th</sup> intake quintiles differed almost significantly ( $p = 0.064$ ), and among females, the 1<sup>st</sup> quintile differed almost significantly ( $p = 0.065$ ) and the 2<sup>nd</sup> quintile differed significantly ( $p = 0.034$ ) from the 5<sup>th</sup> quintile in IMT. Furthermore, a significant positive linear trend occurred between P density of the diet and IMT among all subjects ( $p = 0.039$ , contrast analysis), and among females an almost significant positive linear trend was present ( $p = 0.067$ , contrast analysis). No significant differences were found among males ( $p > 0.05$ ).

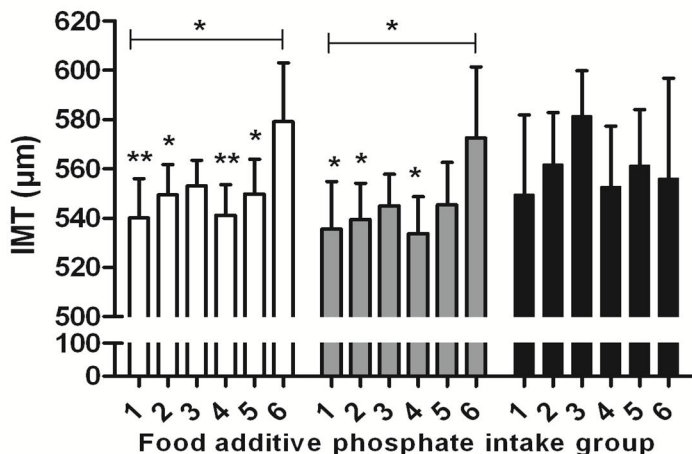


**Figure 15.** Mean carotid intima-media thickness (IMT) with upper limit of 95% confidence interval in P density quintiles for ALL SUBJECTS (WHITE SCALE,  $p$  for all 0.289, ANCOVA), for FEMALES (GRAY SCALE,  $p$  for all 0.231, ANCOVA), and for MALES (BLACK SCALE,  $p$  for all 0.432, ANCOVA). Significant differences from the highest P density quintile (ANCOVA) / statistically significant trends (contrast analysis) between P density and IMT are indicated with asterisks (\* $p < 0.05$ ) (between all other groups  $p > 0.05$ ).

### ***Food additive phosphate intake***

The population was divided into six groups based on FAP intake scores. No significant association between FAP intake and IMT was observed (for all  $p = 0.097$ , for females  $p = 0.267$ , ANCOVA adjusted for sex [when analyzing all subjects], age, LDL-C/HDL-C ratio, smoking status, and IMT sonographer class) (Figure 16). Nevertheless, comparison of the intake groups revealed that among all subjects, IMT was higher with higher FAP intake, and the 6<sup>th</sup> intake group differed significantly from almost all other groups (1<sup>st</sup>  $p = 0.009$ , 2<sup>nd</sup>  $p = 0.032$ , 3<sup>rd</sup>  $p = 0.052$ , 4<sup>th</sup>  $p = 0.006$ ,

5<sup>th</sup>  $p=0.038$ ); this was also the case among females (1<sup>st</sup>  $p=0.037$ , 2<sup>nd</sup>  $p=0.045$ , 3<sup>rd</sup>  $p=0.086$ , 4<sup>th</sup>  $p=0.019$ , 5<sup>th</sup>  $p=0.113$ ). In addition, a significant positive linear trend existed between FAP intake and IMT among all subjects ( $p=0.022$ , contrast analysis) and among females ( $p=0.045$ , contrast analysis). Among males, no significant differences were found ( $p>0.05$ ).



**Figure 16.** Mean carotid intima-media thickness (IMT) with upper limit of 95% confidence interval in FOOD ADDITIVE PHOSPHATE (FAP) intake groups for ALL SUBJECTS (WHITE SCALE,  $p$  for all 0.097, ANCOVA), for FEMALES (GRAY SCALE,  $p$  for all 0.267, ANCOVA), and for MALES (BLACK SCALE,  $p$  for all 0.372, ANCOVA). Statistically significant differences from the highest FAP intake group (ANCOVA) / statistically significant trends (contrast analysis) between FAP intake and IMT are indicated with asterisks (\* $p<0.05$ , \*\* $p<0.01$ ) (between all other groups  $p>0.05$ ).

## **6. Discussion**

### **6.1. Bioavailability of phosphorus in foodstuffs**

#### **6.1.1. Reliability of the analysis method for *in vitro* digestible phosphorus**

According to measurement uncertainty calculation, the analysis method for *in vitro* digestible P seems to be trustworthy. The method originally developed by Asp et al. (1983) for the analysis of dietary fiber was later modified by Ekholm et al. (2000, 2003). Here, some modifications for the determination of DP were made. The rationale of the method was to mimic the processing of foodstuffs in the alimentary tract using alimentary enzymes with changes in pH and processing temperatures. Based on the calculations, the impact of measurement uncertainty on the DP results was 7.4%. In Study II, the proportion of DP in some foodstuffs exceeded 100%, which may be explained by the measurement uncertainty. However, *in vitro* methods only yield suggestive information on bioavailability, since the interaction between alimentary tract and food cannot be measured, neither the circulation exists (Sandberg 2005). This naturally increases the uncertainty in the *in vitro* results and limits the extrapolation of these results to *in vivo* circumstances, and thus, validation *in vivo* is needed (Miller and Berner 1989).

#### **6.1.2. Effect of processing on *in vitro* digestible and total phosphorus contents in cereals**

In the present study, different rye breads were found to have similar proportions of DP (68-78 %), while in rye dough, the proportion of DP was almost 100%. In some earlier studies, the phytic acid content in rye bread was almost reduced to zero during processing (Larsson and Sandberg 1991, Fretzdorff and Brümmer 1992), which probably also

occurred in our study, but during baking of rye bread some P of dough may have become insoluble for unknown reasons, because the DP contents in bread were lower than in dough.

The results resemble those of previous studies in which phytates were mostly degraded in sourdough rye bread (Plaami and Kumpulainen 1995), and are also compatible with Karp et al. (2007) study, who found that the intake of unfermented cereals did not increase S-Pi concentration compared with other P sources, such as cheese and meat, indicating a lower bioavailability of P in cereals. Here, the bakery breads did not seemingly differ in P contents compared with home-baked bread, even though they might not have been leavened for as long time as home-baked breads. Rye bread in Finland is prepared by fermenting the dough, traditionally without yeast. However, exact information about fermentation times of the bakery products is not available. When compared to other analyzed cereals, the TP contents in flour were much higher (about 300 mg/100g) than in doughs or breads, which, however, is logical because doughs and breads also contain ingredients besides flour.

TP contents in wheat samples were lower than in rye products because also fiber and whole-grain contents were lower. In the studied wheat samples, the mean proportion of DP was about 50%, and it increased with processing especially in soured wheat bread. As shown in t-test, significant differences were present between flour-dough, flour-bread, and dough-bread pairs, except for flour-unsoured dough pair. Naturally, wheat flour had the highest contents of total P (about 110 mg/100 g) because no water or other ingredients were added as in doughs and breads. When comparing the amounts of DP in dough and bread originating from 100 g of flour, DP in wheat sourdough and soured wheat bread increased significantly relative to flour or unsoured dough and bread. The soured wheat bread contained a small amount of rye sourdough, but it likewise seemed not to affect the results, although souring is known to decrease phytic acid content (le Francois 1988), while in a previous study adding of rye sourdough increased phytic acid hydrolysis in oat-containing doughs (Larsson and Sandberg 1991). These results support the studies of



Reale et al. (2004), in which the fermentation time of yeast-containing bread was not long enough to degrade phytates: neither changes in DP contents between flour and yeast-fermented dough were seen here. The added amount of yeast was probably too low to cause an increase in the DP content of the breads, although yeast contains a small amount of phytase (Türk et al. 1996).

Barley porridge, which was baked in the oven for three hours, contained a much higher percentage of DP than barley grits, indicating that a long hydrothermal processing causes degradation of phytate and thus, increases inorganic, digestible P contents (Sandberg 2002). The DP content in that amount of barley grits needed for 100 g porridge was more than double when comparing to the original DP content of grits. Nevertheless, one must take into account that barley porridge is usually made with milk, which includes Ca. Ca prevents phytate degradation, at least in doughs (Türk and Sandberg 1992), and milk also contains P, which would also affect the results. Thus, the results of milk-based porridge would probably be different.

### **6.1.3. *In vitro* digestible and total phosphorus contents in other plant-based foodstuffs**

Both TP and DP contents varied widely in food groups analyzed. Protein content mainly explains the differences in TP contents in regular products, but the proportion of DP can be affected by food processing in food containing phytic acid (as seen in Study I), or by the use of P-containing food additives. In cereals, seeds, and legumes, phytate-derived P accounts for the majority of TP (Reddy 2002), but it needs to be hydrolyzed to release inorganic Pi in a form available for absorption in the body. Although phytase activity in human gut is very low (Iqbal et al. 1994), different food processing methods can hydrolyze phytic acid (Plaami 1997). Thus, more P is likely to be digestible from processed (i.e. fermented, soaked) plant-derived foodstuffs than from raw or unprocessed foods.

Of the sweet bakery products, sweet buns (leavened with yeast) and cookies (containing no FAPs) had a P content similar to that in white wheat bread (analyzed in Study I). However, industrially prepared muffins containing FAP (baking powder) differed from other wheat products; the percentage of DP was almost 100%. TP content of muffins was similar to that of rye bread, but DP content was higher than in any breads. An average muffin and sweet bun both weight about 50 g; if a muffin with FAP is chosen, the amount of DP is 100 mg, but if the yeast-leavened sweet bun was chosen, the amount of DP is only 30 mg. Therefore, persons who consume large amounts of FAP-containing bakery products may ingest substantial amounts of bioavailable P.

Of the foods analyzed in this study, the proportion of DP from TP was lowest in legumes and seeds (6-42% of TP). Earlier results have suggested that bioavailability of P in beans is only 25% (Uribarri 2007). Findings of the current study, by contrast, indicate DP contents in legumes to be on average higher than this. However, similarly to lentils, beans, and peas, sesame seeds with hull had a low DP content (42 mg/100 g). This is in line with the results of an earlier short-term controlled study, where whole sesame seeds did not affect the concentrations of S-Pi indicating them to be poor sources of bioavailable P (Kärkkäinen et al. 1997). Nevertheless, the cooking time of lentils, green beans, and peas is relatively short, and these products do not require soaking before cooking (except dry peas), which would decrease phytate contents and release inorganic Pi (Sandberg 2002). Thus, it is unlikely that a large proportion of phytate is hydrolyzed during processing, and therefore, DP content in lentils, green beans, and peas would not rise notably, if at all, when these products are cooked. Nevertheless, to evaluate the bioavailable P in legumes requiring long soaking and cooking times (such as soy beans and chick peas), more analyses of the effects of processing on DP contents are needed.

In beer and cola beverages, all P was digestible. This result is in line with previous results on high absorbability of P from food additives such as

phosphoric acid in cola drinks which appears to absorb completely in the intestine (Bell et al. 1977, Karp et al. 2007). TP contents in both cola drinks and beer were comparable with values published earlier (Murphy-Gutekunst 2005, Institution for Health and Welfare 2011). In beer, P originates from the grain used in preparing the product; in Finland, this is most often barley. Based on these results, it seems that during malting and other processing of grain when preparing beer, P is efficiently released from the grain to a highly digestible form, resulting in a high proportion of DP. Processing of beer also includes fermentation with yeast, which is known to contain phytase, and thus, the ability to degrade phytate to bioavailable P (Türk et al. 2006). However, in beer, as in some cola beverages the amount of DP exceeded the amount of TP. This may be explained by the uncertainty of the analysis method under circumstances of low P content; the calculated measurement uncertainty for the DP analysis method in Study I was 7.4%. Thus, at small concentrations of DP (beer 22 mg/100g, colas 10-16 mg/100g), a 1-2 mg change within the DP contents can be explained by the variability of the method. Nevertheless, even though the amounts of P in these beverages are not very high, persons consuming beer or cola drinks in abundance may ingest a considerable amount of DP in these drinks. This may be important because replacing milk (containing Ca and natural P) with cola beverages and increasing beverage consumption in the general population can have adverse effects on Ca and bone metabolism (Fernando et al. 1999, Kristensen et al. 2005, Tucker et al. 2006).

Based on the results of Studies I and II, *in vitro* DP content in different rye breads can vary substantially, most likely depending on the amount and proportion of rye flour and crushed rye grain, the content of other grains or potato, the use of yeast, and the length of fermentation (Türk et al. 1996, Sathe and Venkatachalan 2002). Further, the grounding stage of the flour generally results in differences in P contents; DP content of white wheat bread analyzed in Study I was lower than DP content of rye or mixed-grain bread in Study II. In practice, the differences in the amounts of P from foodstuffs can easily be demonstrated by calculating regular portions of P in DP and TP. If one person eats 5 slices of bread

(here: small rye bread containing potato 1 from Study II, 33 g/each) per day, the amount of P intake varies markedly depending on whether calculated by TP or DP; TP from bread is 317 mg, and DP is 147 mg. The amount of P calculated as TP is double that of DP. If the same amount of white wheat bread (Study I) is chosen, the amount of TP would be 136 mg, and DP 70 mg. This shows that depending on the calculation method to take into account TP or DP, P intake can vary widely. At least in CKD patients, this may be relevant because in practice they must follow a P-restricted diet and food choices can have a real effect on P intake and diversity of the diet. However, use of different porridges made of flakes (oat, rye, mixed cereals) is also common in Finland (Helldán et al. 2013b), and analysis of their DP contents should be considered.

#### **6.1.4. Conclusions about phosphorus bioavailability in analyzed foodstuffs**

The *in vitro* DP content in processed cereals was less than the in unprocessed cereals, confirming the hypothesis. This is probably because during processing of foodstuffs phytate was degraded to lower inositol Pi's and the same process formed inorganic Pi, which increased the DP content (Sandberg 2002). The products were processed under circumstances where both enzymatic and non-enzymatic degradation can occur (low pH, high temperature, presence of yeast, hydrothermal processing) (Sandberg 2002, Sandberg and Andlid 2002); some of the doughs were soured and some were leavened with yeast and baked which should increase the inorganic Pi contents.

DP contents among other studied plant-based foodstuffs seemed to differ substantially among different foodstuffs. Despite high TP content, legumes and seeds may be relatively poor P sources of bioavailable P. Results support previous understanding of the effective absorbability of P from P additives. Bakery products containing baking powder may substantially increase intake of DP. Thus, these bakery products can be an important source of highly bioavailable, in addition to meat products,

processed cheeses, and cola beverages. However, the results of DP provide only suggestive data on P bioavailability, and thus, should be confirmed with an *in vivo* method. If DP analysis is proven a valid method for evaluating P availability, it could be used to estimate the bioavailability of P in eg. P-restricted diets.

These results indicate that different P sources their bioavailability should be taken into account more seriously in research on P intake and health outcomes. The original aim was to use the results of bioavailable P contents of Studies I and II in assessing the associations of dietary P with CVD risk factors (Study III). This, however, proved to be impossible because only particular foodstuffs among the thousands included in food composition databases were analyzed. Nevertheless, in Study III, we were able to analyze the data concerning both total P sources, and the potentially more detrimental FAP-containing products.

## **6.2. Dietary phosphorus as a potential cardiovascular risk factor**

### **6.2.1. Total phosphorus intake and cardiovascular health**

This study demonstrated that high dietary P intake should be taken into account as a potential cardiovascular risk factor in the general population. Positive linear trends in associations among P density of diet and IMT were found. IMT of females was significantly higher in the highest TP intake quintile than in the lowest intake quintile, and an almost significant linear trend emerged between TP intake and IMT. Here, attention was paid to TP intake, which, when divided by energy intake describes the P density of the diet, excluding energy intake as a potential confounding factor. In the study of Chang et al. (2014a) in particular high P density of the diet was associated with adverse mortality outcomes. The results support the hypothesis that in addition to elevated S-Pi concentration, also higher dietary P intake may be a cardiovascular risk factor in terms of high IMT in a normal, healthy population. Individuals with low glomerular filtration rate (eGFR <60 ml/min) were excluded, and thus, the possible effects of improper kidney function did not affect the results. Earlier data on health effects of high P intake on the normal population are scarce, and most studies carried out on P intake have focused on bone health (Calvo 1993, Kärkkäinen and Lamberg-Allardt 1996, Kemi et al. 2006, 2009, 2010).

The subjects, who participated in the present study, were middle-aged and on average overweight (mean BMI 26.8 kg/m<sup>2</sup>). Dietary P intake in study subjects was slightly higher than the habitual P intake in the national FINDIET 2012 Study (females 1532 mg/d vs. 1369 mg/d, males 1795 mg/d vs. 1694 mg/d) (Helldán et al. 2013a). While marked variation in TP intake can be seen between the quintiles (smallest intake quintile ca. 1100 mg/d, and highest ca. 2300 mg/d), on average all subjects exceeded the recommended intakes (National Nutrition Council 2014). P density of the diets was similar to mean intakes in the FINDIET 2012 Study (females

195 mg/MJ/d vs. 197 mg/MJ/d, males 192 mg/MJ/d vs. 187 mg/MJ/d) (Helldán et al. 2013a). The mean Ca intake met the recommendations (National Nutrition Council 2014) and vitamin D intake and the S-25(OH)D concentrations were satisfactory, especially taking into account that the study was carried out during the dark season. Thus, the study population may have had a better Ca balance and nutritional status than other populations because of the adequate intakes of Ca and vitamin D (e.g. Welch et al. 2009).

However, human data on dietary P intake and CVD risk factors are limited. Acute high P intake has decreased endothelial function of the vessels (Shuto et al. 2009), and high dietary P intake was associated with left ventricular hypertrophy among women but not among men (Yamamoto et al. 2013). Recently, in the NHANES cohort, dietary P intake >1400 mg/d and P density of diet >0.35 mg/kcal (corresponding to 146 mg/MJ) were associated with all-cause mortality, and high P density (>0.35 mg/kcal) was associated with CVD mortality (Chang et al. 2014a). In that study, also low P density of diet (<0.35 mg/kcal) was associated with increased mortality risk. Notable in these above-mentioned studies is that the P intakes associated with adverse health effects, are similar to or lower than the average intakes in Finland (Helldán et al. 2013a). The NHANES Study produced much discussion about P sources and their bioavailability (McCarthy 2014); the results of Studies I and II contribute to this discussion.

### **6.2.2. Food additive phosphorus intake and cardiovascular health**

Here, the research question was addressed separately for TP intake and FAP intake because FAP is more absorbable in the intestine than natural P (Bell et al. 1977, Karp et al. 2007). As noted in Study II, and consistent with an *in vitro* study by Karp et al. (2012), the amounts of DP are higher in foodstuffs containing FAPs than products without FAPs. As mentioned earlier, the contribution of FAP to TP are mainly unknown and amounts of FAP in foodstuffs vary greatly between manufacturers. In the current

study, positive linear trends in association between FAP intake and IMT were found when analyzing all subjects and females.

Previous data on FAP intake are scarce and studies on health outcomes of FAP are mainly concerned on bone health (Fernando et al. 1999, Kristensen et al. 2005, Tucker et al. 2006, Karp et al. 2007, Kemi et al. 2009, Karp et al. 2013). In the earlier-mentioned studies on dietary P and CVD (see Section 6.2.1.), FAP intake data was unavailable. Here, FAP-containing meat products, processed cheeses, and cola beverages were included in calculations, and significant differences in IMT emerged between subjects having high and those having low FAP intake. Nevertheless, in nutrient values based on food composition databases, FAPs may not have been taken into account due to the calculated nutrients values which are not based on chemically analyzed values. Many studies conclude that TP content in, for example, meat products can be twice as high as that calculated from the nutritional programs based on food composition databases, since analyzed data on FAP content is missing in the current databases (Oenning et al. 1988, Calvo et al. 2014). This can also uncertainty in results of the current study.

### **6.2.3. Serum phosphate and other regulators of phosphate metabolism and cardiovascular health**

A few studies have been conducted to assess the associations between elevated S-Pi concentrations and IMT or vascular calcification, but this study is one of the first to investigate the association of dietary P with IMT. Ruan et al. (2010) found that even in young persons S-Pi was associated with higher cIMT. Onufrak et al. (2008), in turn, showed that S-Pi was associated with IMT among males but not among females. In a Korean study (Park et al. 2010) low S-Pi was associated with less coronary calcification, and Foley et al. (2009) found high S-Pi to be associated with coronary calcification. Tuttle and Short (2009) showed in a longitudinal design in adult population that S-Pi concentrations independently predicted coronary artery calcification. Numerous other



data on association of high S-Pi and CVD risk also exist (Tonelli et al. 2005, Dhingra et al. 2007, Foley et al. 2008, Ix et al. 2009, Kendrick et al. 2010, Larsson et al. 2010, Sim et al. 2013), but the findings of Taylor et al. (2011) showing no association of plasma Pi with development of coronary heart disease among males are not in accord with the others.

Interestingly, in the current study, S-Pi was highest with the lowest FAP intake scores among all subjects. Earlier cross-sectional studies have shown that dietary P intake correlates only weakly with S-Pi (Mataix et al. 2006, de Boer et al. (2009). Uribarri (2013) suggests that due to the high circadian variation of S-Pi and very weak correlation between P intake and S-Pi in cross-sectional studies, peak S-Pi concentration after oral Pi load, in addition to mean 24-h S-Pi concentrations, could be better indicators of potential adverse health effects. Ellam and Chico (2012) state that high bioavailability of P in Western diets may cause high post-prandial Pi peaks, and in healthy humans, oral Pi supplementation has been noted to cause S-Pi to rise to the upper level of the normal range (Kärkkäinen and Lamberg-Allardt 1996, Kemi et al. 2006, Karp et al. 2007). However, in our study, we were only able to measure fasting S-Pi concentrations. Thus, in future research, more resources should be directed to more extensive S-Pi assessments, not only relying on fasting concentrations. Also measuring urine Pi excretion would be valuable (Karp et al. 2007, Morimoto et al. 2014); however, 24-h urine collection is laborous to carry out and can only be used for subjects with normal renal function.

The main factors involved in P metabolism, namely FGF23, PTH, and calcitriol, have been found to be independently adversely associated with cardiovascular health (Uribarri and Calvo 2014). Although dietary P load has been shown to increase FGF23 concentration during a long exposure (Block et al. 2013), one failed to find any association between P-FGF23 concentration and dietary P intake in the current study, probably due to the cross-sectional study design. Also the analysis of the C-terminal fraction of FGF23 may explain the lack of the correlations in the current study; as mentioned earlier, intact FGF23 has been found to respond

better to dietary P load (Burnett et al. 2006). FGF23 has been speculated to participate in the effects of P on CVD, but the mechanisms are hypothetical and mainly unknown (Kendrick et al. 2011). High FGF23 has been associated with CVD risk factors (Gutiérrez et al. 2011) and CVD itself (Dalal et al. 2011) but results of studies are discordant (Taylor et al. 2011, Schoppet et al. 2012), and to date, no studies have focused on FGF23 and IMT in the general population.

PTH concentrations increase acutely after dietary P load (Vervloet et al. 2011, Block et al. 2013). However, in the present study dietary P variables were associated with IMT, but no correlation between serum iPTH concentrations and IMT was observed (data not shown). A meta-analysis of 15 prospective studies showed that higher PTH concentrations were associated with increased risk of CVD events (van Ballegooijen et al. 2013). However, with regard total CVD events (10 studies included in the analysis) only three of the studies showed a positive association between PTH and CVD, the rest reporting no association (van Ballegooijen et al. 2013). In the present study, S-Pi, FGF23 and iPTH analyses concerning IMT were not adjusted for potential covariates because they were not main parameters of the study but the data will be further analyzed in other manuscripts. However, the results should be interpreted with caution: more investigation on these surrogate markers of P intake and cardiovascular health are warranted.

#### **6.2.4. Conclusions about dietary phosphorus as a potential cardiovascular risk factor**

The results show significant linear trends in the associations among P density of the diet, FAP intake, and carotid IMT in a healthy, middle-aged Caucasian population. Based on these results, high dietary P intake should be further investigated due to its potential association with CVD risk factors in the general population, not only in renal patients. Furthermore, prospective, or even better, long-term intervention studies are required to

evaluate the possible impact of dietary P burden on risk of CVD. Also the regulators of P metabolism should be taken into account.

## 6.3. Strengths and limitations of the studies

### 6.3.1. Food studies (Studies I and II)

In the method for DP analysis, the processing of the food in the alimentary tract was mimicked, but this *in vitro* method do not take into account the interaction between the alimentary tract and food or components of food (Sandberg 2005), and therefore the results on DP need further validation with an *in vivo* method. For example, the transit time in the alimentary tract depends on the content of the meal, and absorption capability in the different parts of the alimentary tract varies. Moreover, the produced alimentary enzymes differ in contents and concentrations depending on the composition of ingested food, neither was the circulation present in this method. This naturally causes uncertainty in extrapolating the *in vitro* results to *in vivo* circumstances.

The ICP-MS and ICP-OES methods for total P analysis are reliable and well validated (PerkinElmerSciex Instruments 2001). The differences in results for P contents in breads between Studies I and II can be partly explained by sampling differences. In Study I, the focus was on the effects of processing on P contents in particular foodstuffs, while in Study II the sampling was ensured to be representative of the most popular trademarks in each food category, and the food consumption data originated from a population study in Finland. Nevertheless, because Study I was a methodological study, the samples were not chosen to be generally representative; the main aims were to develop a new analysis method and to examine the effect of baking processes on the DP contents.

The percentage of DP from TP was in some foodstuffs more than 100%, which may be explained by the variance in the uncertainty of the method, at least at low P concentrations. Measurement accuracy was, however, improved by extraction of fat from high-fat-containing foodstuffs. In the present studies, only plant-based foodstuffs were analyzed but the same

method has been used for analysis of animal-based foodstuffs in the study by Karp et al. (2012).

### **6.3.2. Human study (Study III)**

Here, a large variety of biomarkers were analyzed and extensive background data were available. Further, the study subjects were homogeneous in age and the females were in specified state of menopause (premenopausal). The potential effects of impaired kidney functions were taken into account by excluding persons with  $eGFR < 60$  ml/min. In large studies where the main focus is not nutritional, food intake data may have been collected less accurately than in our study. Nevertheless, among the study subjects Ca intake was high, and serum 25-hydroxy vitamin D status was fairly satisfactory, both of which interact with P metabolism and may hide adverse effects of high P intake. One limitation of the study is its cross-sectional design, which prevents an evaluation of causality. The number of males (176 vs. 400 expected) may have been too small to show statistically significant results.

One problem with dietary intake calculation programs is that the analyzed TP content, for example in meat products, can be twice as high as that based on food composition databases due to a lack of analyzed data for FAP content (Calvo et al. 2014). Here, the food intake (except FAP intake) was assessed only with self-reported food records (3 days); however, the records were checked by researchers, in an attempt to improve the quality of the data. In addition, 400 new recipes were introduced to the nutrient calculation program to ensure more accurate assessment of P intake and other nutrients. However, the database used is based on Fineli<sup>®</sup>, which has limited information on FAPs. Thus, it is important to have data on consumption of FAP-containing foodstuffs measured by a validated FFQ. Also different processing methods for foods actually consumed and the subjects' former diets may have affected the associations of dietary P with other measured parameters. Food records and FFQs were not repeated, and reveal the only current dietary intake and it is difficult to know

whether the dietary intake method used is indicative of the usual diet. The method by which FAP intake is calculated can also be criticized; in the present study FAP intakes were not able to calculate precisely, and only FAP-containing meat products, processed cheeses, and cola beverages were included in the FAP intake calculations, but, however, information on exact amounts of FAPs in foodstuffs was not available.

IMT measurement by sonography is a reproducible, rapid, inexpensive, and radiation-free technique to evaluate the condition of arteries and risk of CVD (Mookadam et al. 2010, O'Leary and Bots 2010). Here, the population was middle-aged, and the age group was chosen based on literature suggesting that some changes in vessels probably already occur by this time. However, it was not able to distinguish between carotid intima and media layers of the vessels, thus, one cannot be sure whether this calcification occurs in the intima, media, or both. That leaves the effect of the P separately on intima and media unknown. More assessments, e.g. measurement of endothelial function of the vessels, would have provided valuable additional information.

## 7. Conclusions and future perspectives

This thesis aimed to provide new insights into the potential health risks related to P intake in Finland by producing new information on the bioavailability of P from foodstuffs commonly used in Finland in terms of *in vitro* digestible P. Due to increased consumption of processed meals, it is important to pay attention to the sources of bioavailable P. Furthermore, the study yielded population-based data on the associations of dietary P, especially food additive P, and vascular calcification in Finland. Cardiovascular diseases are common causes of death in Finland, and the information about the potential detrimental role of dietary P in these diseases is highly important.

Specific findings of the studies were as follows:

**Food studies (Studies I and II):** Information on *in vitro* digestible P contents in differently processed cereals, as well different plant-based foodstuffs was attained. Also a new reliable analysis method was tested. Despite high total P content, cereals, legumes and seeds may be relatively poor sources of bioavailable P, even though processing of the cereals increases DP contents. Results support previous understanding of the effective absorption of P from additives. The use of food additive phosphate-containing products may substantially increase the intake of highly bioavailable P. The results of *in vitro* digestible P should, however, be confirmed with an *in vivo* method.

**Human study (Study III):** High P density of the diet i.e. total dietary P intake relative to energy intake, and high food additive phosphate intake were associated with carotid intima-media thickness in healthy middle-aged Finnish adults. However, due to the cross-sectional design, causal effects cannot be shown. Based on these results, high dietary P should be further investigated for its potential association with cardiovascular disease risk factors in the general population, not only in renal patients. The relationship between high P intake and vascular calcification requires also further elucidation.

In conclusion, the present results highlight the importance of the source of P when analyzing P intake and P burden in a population. Food additive phosphate-containing foodstuffs have higher contents of *in vitro* digestible P, and also processing increases the digestible P contents. Both intervention and follow-up studies are warranted on different populations before final conclusions about the harmfulness of high P intake on cardiovascular health in the general population can be drawn. Data on use of food additive phosphates in the food industry and updated information on P contents in foodstuffs are also needed.



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## APPENDIX

**Table 1.** Wet digestion program for total and in vitro digestible phosphorus analysis in Study I.

Phase	Total phosphorus		Digestible phosphorus	
	Temperature (°C)	Time (h)	Temperature (°C)	Time (h)
1	70	3	70	6
2	90	0,5	90	1,5
3	110	0,5	110	1,5
4	130	3,5	130	6
5	145	0,5	-	-
6	160	0,5	-	-

**Table 2.** Information on home-baked samples in Study I. Baking temperature was 200 °C, except for barley porridge, which was baked at 120 °C.

Sample	Ingredients	Leavening time (h)	Baking time (min)
Wheat bread	White wheat bread flour (ash content 0.6%), water, yeast, NaCl	0.75	25
Sourdough wheat bread	White wheat bread flour (ash content 0.6%), water, yeast, rye sourdough, NaCl	20	25
Whole-grain rye bread	Whole-grain rye flour (ash content 1.6%), water, rye sourdough, NaCl	23	60
Barley porridge	Barley grits, water, salt	-	180

NaCl = sodium chloride.

**Table 3.** The absolute amounts of in vitro digestible phosphorus (DP) and total phosphorus (TP) contents within measurement uncertainty of the analyzed products.

	TP (mg/100g)	DP (mg/100g)
	Mean ± measurement uncertainty	Mean ± measurement uncertainty
Rye flour	300.8±19.2	135.0±10.0
Rye sourdough	160.7±10.2	131.6±9.7
Bakery whole rye bread	203.8±13.0	145.4±10.8
Bakery rye bread containing wheat and potato	186.6±11.9	128.7±9.5
Home-baked rye bread	171.9±11.0	134.4±10.0
Wheat flour	116.7±7.4	50.2±3.7
Unsoured wheat dough	76.9±4.9	42.8±3.2
Unsoured wheat bread	82.2±5.2	38.1±2.8
Soured wheat dough	79.4±5.1	47.7±3.5
Soured wheat bread	81.5±5.2	39.0±2.9
Bakery wheat bread	82.5±5.3	42.3±3.1
Barley grit	153.7±9.8	43.8±3.2
Barley porridge	29.0±1.9	20.1±1.5